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(71) Applicant *(for all designated States except US)*: GENENCOR INTERNATIONAL INC. [US/US]; 180 Kimball Way, South San Francisco, CA 94080 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: WARD, Michael [GB/US]; 381 Myrtle Street, Half Moon Bay, CA 94019 (US). SHOEMAKER, Sharon, P. [US/US]; 3173 Burbank Drive, Fairfield, CA 94533 (US). WEISS, Geoffrey, L. [US/US]; 457 Buena Vista Avenue, San Francisco, CA 94117 (US).

(74) Agent: PASSE, James, G.; Burns, Doane, Swecker & Mathis, George Mason Building, Washington and Prince Streets, P.O. Box 1404, Alexandria, VA 22313-1404 (US).

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(54) Title: TRICHODERMA REESEI CONTAINING DELETED AND/OR ENRICHED CELLULASE AND OTHER ENZYME GENES AND CELLULASE COMPOSITIONS DERIVED THEREFROM

(57) Abstract

A process for transforming the filamentous fungus *T. reesei* which involves the steps of treating a *T. reesei* strain with substantially homologous linear recombinant DNA to permit homologous transformation and then selecting the resulting *T. reesei* transformants. Transformants made by this process are disclosed, as well as cellulase compositions prepared via the transformed strains.

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TRICHODERMA REESEI CONTAINING DELETED AND/OR ENRICHED CELLULASE AND OTHER ENZYME GENES AND CELLULASE COMPOSITIONS DERIVED THEREFROM

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a process for transforming the filamentous fungus Trichoderma reesei; to transformation of Trichoderma reesei with homologous DNA including a selectable marker for transforming Trichoderma reesei; to deletion of Trichoderma reesei genes by transformation with linear DNA fragments of substantially homologous DNA; to insertion of Trichoderma reesei genes by transformation with linear DNA fragments of substantially homologous DNA; to useful fungal transformants produced from Trichoderma reesei by genetic engineering techniques; and to cellulase compositions produced by such transformants.

State of the Art

Cellulases (i.e., the cellulase system) are enzyme compositions which hydrolyze cellulose (ß-1,4-D-glucan linkages) and/or its derivatives (eg., phosphoric acid swollen cellulose) and give as primary products glucose, cellobiose, cellooligosaccharide, and the like. A cellulase system produced by a given microorganism is comprised of several different enzyme classifications including those identified as exo-cellobiohydrolases (EC 3.2.1.91) ("CBH"), endoglucanases (EC 3.2.1.4) ("EG"), and ß-glucosidases (EC 3.2.1.21) ("BG") (Schulein, M., 1988). Moreover, these classifications can be further separated

into individual components. For example, multiple CBH-type components and EG-type components have been isolated from a variety of bacterial and fungal sources including Trichoderma reesei, hereinafter T. reesei, which contains at least two CBH components, i.e., CBHI and CBHII, and at least three EG components, i.e., EGI, EGII and EGIII components. T. reesei has also been referred to in the literature as Trichoderma longibrachiatum Rifai (Cannon, P.F., 1986, Microbiol. Sci. 3 pp. 285-287).

It is noted that EGII has been previously referred to by the nomenclature "EGIII" by some authors but current nomenclature uses the term "EGII". In any event, the EGII protein is substantially different from the EGIII protein stated herein in its molecular weight, pI, and pH optimum.

The complete cellulase system comprising CBH, EG, and BG components is required to efficiently convert crystalline cellulose to glucose. Isolated components are far less effective, if useful at all, in hydrolyzing crystalline cellulose. Moreover, a synergistic relationship is observed between the cellulase components CBH, EG and BG on crystalline cellulose. That is to say the effectiveness of the complete/whole system to solubilize cellulose is significantly greater than the sum of the contributions from the isolated components. It also has been shown that CBHI- and CBHII-type components derived from either T. reesei or P. funiculosum act synergistically in solubilizing cotton fibers (Wood, 1985). Moreover, it has been disclosed that CBHI (derived from T. reesei), by itself, has the highest binding affinity but the lowest specific activity of all forms of cellulase components

component which may account for the synergy of the combined components.

The mechanism by which crystalline cellulose is depolymerized by the cellulase enzyme system has not been completely elucidated. Without being limited to any theory, there is increasing evidence that the endoglucanases and exo-cellobiohydrolases interact in binding and subsequent hydolysis and that the mechanism is more complicated than has been thought. That is, not only do endoglucanases provide by their action more non-reducing chain ends for exo-cellobiohydrolases but there also appears to be some interaction between the various enzyme components in binding and subsequent hydrolysis. There is preferential hydrolysis at regions of low crystallinity and often accessibility may be the limiting factor in the depolymerization reaction. As separate enzymes, the endoglucanases act on internal linkages (with higher rates of reaction on cellulose regions of low crystallinity) and give as principle soluble products, cellobiose, glucose and cellooligosaccharides. The exo-cellobiohydrolases, in contrast, act from the non-reducing end of the cellulose polymer chains to give cellobiose as the principle product. β-glucosidases do not act on the polymer but act on soluble cellooligosaccharides from the non-reducing end to give glucose as the sole product.

Cellulase is also known in the art to be useful in detergent compositions either for the purpose of enhancing the cleaning ability of the composition or as a softening agent. When so used, the cellulase will degrade a portion of the cellulosic material, e.g., cotton fabric, in

the wash which in one manner or another facilitates the cleaning and/or the softening of the cotton fabric. While the exact cleaning and softening mechanisms of cotton fabrics by cellulase are not fully understood, the cleaning and softening of cotton fabrics by cellulase has been attributed to different components found in the cellulase. For example, U.S. Patent Application Serial No. 07/422,814 (abandoned in favor of continuation application U.S. Patent Application Serial No. 07/686,265), incorporated herein by reference, discloses that excellent cleaning of cotton fabric can be achieved without degrading the cotton fabric by using cellulase compositions enriched in CBHI-type components; whereas International Application Publication No. WO 89/09259, also incorporated herein by reference, discloses that improved softening of cotton-containing fabrics can be achieved by using a cellulase composition enriched in an endoglucanase-type component meeting the criteria defined therein. Therefore, since different cellulase components influence the cleaning and softening effects it would be desirable to isolate these components in pure form and to prepare detergent compositions therefrom enriched in one or more particular components.

One means of isolating such enriched cellulase components is by purification techniques. However, purification from the fermentation broth via chromatographic techniques, electrophoretic techniques and the like, is typically time consuming and expensive. Construction of microbial strains, via genetic techniques, which are depleted or enriched in one or more cellulase components would greatly enhance the commercial utility of cellulase.

In this regard, selected strains of the imperfect fungus T. reesei, as well as other strains of fungus, are well known for the high volumetric productivity with respect to the production of extracellular cellulase. Indeed, T. reesei appears to be the host of choice for transformation and production of cellulase because of its high protein secretory capacity.

Xylanase is known in the art to be useful in a number of commercial processes. The xylanase enzymes are generally used to hydrolyze and/or modify xylan containing polymers which are associated with hemicellulose and other plant polysaccharides. Xylanase enzymes have been found to be useful in a variety of applications including but not limited to the bleaching of wood pulps and the modification of cereals and grains for use in baking and the production of animal feeds. Construction of microbial strains, via genetic techniques, to overexpress the xylanase proteins free of cellulolytic enzymes would greatly enhance the commercial utility of xylanase.

Transformation is a known process for transferring genetic material into a host microorganism. This process has been well established in procaryotic systems, but in higher organisms such as eukaryotes, transformation in many instances is still in experimental stages. Transformation in fungi has been limited in part because of the low permeability of the cell wall, which in many instances tends to restrict the uptake of DNA into the host strain. A transformation system in the yeast Saccharomyces cerevisiae recently has been developed by digesting the outer wall of the yeast cells with various

enzymes, thus aiding in DNA uptake in the host. Cloned DNA sequences were introduced into the host and homologous recombination occurred. That is, the plasmid integrated into the genome by recombination between a DNA segment in the genome and a similar DNA segment present on the plasmid. Alternatively, some plasmids are capable of autonomous replication and exist free from the host cell genome in yeast.

It has been further reported that transformation has been attempted in many different types of fungi such as Saccharomyces (Hinnen et al., 1978; Beggs et al., 1978), Neurospora (Case et al., 1979), Podospora (Tudzynski et al., 1980; Stahl et al., 1982), Schizosaccharomyces (Beach et al., 1981), Aspergillus (Ballance et al., 1983), Schizophyllum (Ulrich et al., 1985), to mention a few. However, the transformation methods among the fungi tend to be quite diverse depending on the host strain used and there appears to be no uniform, single method to transform fungal cells. The prior art teaches a diverse number of methods and strategies for transformation of fungal cells, due to the unique characteristics of each fungal species. This is due in part to the fact that DNA access to the host cells, DNA maintenance in the host cell (i.e., as autonomous plasmid or integration into the host cell genome) and gene expression appear to be quite different for each fungal species.

Moreover, it has been further noted that the particular host strain in fungi strongly influences the targeting of DNA integration into the host cell genome achieved in the transformation process. If transformation with cloned or recombinant DNA sequences is achieved

in fungal strains, integration of the DNA sequences into the host strain often occurs at secondary sites rather than at the homologous region of the genome (Case et al., 1979; Case, 1986; Dhawale et al., 1985; Paietta and Marzluf, 1985).

In the past, transformation methods for T. reesei have used foreign DNA in the vector system which contains a selectable marker capable of being incorporated into the host strain. Circular vectors incorporating bacterial plasmid DNA have been used and the selectable marker gene has been derived from another species. For instance, in European Patent Application No. 0,244,234, T. reesei was transformed using selectable markers of argB, trpC or amds from the species Aspergillus nidulans. Also disclosed is the use of pyr4 from the species Neurospora crassa. All of the selectable markers are genes which are heterologous to the host strain and therefore foreign DNA is introduced into the derivative strain.

The insertion of foreign DNA sequences into a strain designed for commercial protein production would require more extensive testing before approval by regulatory organizations than if only homologous DNA were inserted at a known site within the genome. Moreover, the integration of a foreign DNA sequence at non-homologous sites within the host genome could potentially and unpredictably alter the spectrum of proteins secreted by the microorganism and therefore result in an altered product.

Gene deletion by DNA mediated transformation in Aspergillus nidulans has been achieved using a linear fragment of homologous

DNA (Miller et al., 1985). The DNA fragment consisted of Aspergillus nidulans DNA from the argB locus with the central argB coding sequence removed and replaced by the Aspergillus nidulans trpC gene. This DNA was used to transform a trpC- argB+ strain to trpC+. In a certain proportion (30%) of the transformants the DNA integrated at the argB locus in the genome in a predicted manner which caused deletion of the argB gene. The resulting strains were thus trpC+ argB-. However, Miller et al. do not disclose any secreted protein produced by the transformed strains.

In contrast, very similar experiments were performed in an attempt to delete the am gene of Neurospora crassa using the qa-2 gene as a selectable marker (Paietta and Marzluf, 1985). In this species non-homologous integration was extremely common and multiple copies of transforming DNA often became integrated. Although the desired gene deletion was occasionally observed, the authors were unable to observe any examples of the predicted, simple integration of a single, linear DNA fragment at the am locus.

As noted above, transformation of fungi to produce various proteins is often unpredictable. Different methods often are used to transform different strains and the DNA is not always integrated at the designated position in the genome. The selection of a host microorganism is vital in the transformation process. The microorganism must be able to be transformed by integration of recombinant DNA at the homologous region of the genome in at least some fraction of the transformants and seldom with additional integration at secondary sites and be able to produce the desired

protein product in quantities that are commercially marketable. Thus, for the production of different components of cellulases, it would be desirable to use a host microorganism that secretes cellulase enzymes at a significant capacity. As noted above, T. reesei is one such strain. However, it has been recently reported that Trichoderma transformants obtained using a pyr gene as a selectable marker show a high degree of instability in contrast to equivalent transformants of Aspergillus niger and Neurospora crassa (Gruber et al., 1990, Smith et al., 1991). Although T. reesei is the host microorganism of choice, it was unpredictable whether homologous recombination could be achieved in this host fungus.

Accordingly, it is an object of this invention to introduce a homologous gene or gene fragment into strains of the fungus T. reesei to produce derivative strains which are deficient for, and/or which overexpress certain native genes. It is a further object of this invention to create such transformants without the introduction of foreign DNA by the use of a linear fragment of DNA originally derived from T. reesei. These and other objects are achieved by the present invention as evidenced by the summary of the invention, description of the preferred embodiments and claims.

SUMMARY OF THE INVENTION

It has now been discovered that T. reesei can be transformed with linear homologous DNA fragments, excised from plasmids, which can integrate at homologous sites in the genome. Moreover, the derivative strains produced by this transformation method may lack particular genes because of homologous integration of the linear DNA

fragment into a copy of this gene locus within the genome. The transformants produced by the transformation do not contain any foreign DNA and thus secrete proteins, such as cellulase enzymes, that are free of any foreign protein. In addition, the derivative strains produced by this transformation method may overexpress particular genes because of the homologous integration of a linear DNA fragment containing a functional gene into the gene locus of another gene within the genome.

Accordingly, in one of its process aspects, the present invention is directed to a process for transforming T. reesei, which process comprises the steps of:

(a) treating a T. reesei strain with substantially homologous linear recombinant DNA under conditions permitting at least some of said T. reesei strain to take up said substantially homologous linear recombinant DNA and form transformants therewith; and

(b) selecting resulting T. reesei transformants.

In one of its composition aspects, the present invention is directed to novel and useful transformants of T. reesei which can be used to synthesize cellulase compositions, especially cellulase compositions deleted or enriched in one or more components and which produce only homologous proteins.

In yet another composition aspect, the present invention is directed to a fungal cellulase composition derived from the transformed T. reesei strains which is lacking cellulase proteins selected from the

group consisting of one or more CBHI-, CBHII-, EGI-, EGII- and EGIII components which composition is free of heterologous proteins.

In yet another composition aspect, the present invention is directed to a fungal xylanase composition derived from the transformed T.reesei strains which is deleted or enriched in one or more xylanase proteins which composition is free of heterologous proteins.

In a preferred embodiment the present invention is directed towards the preparation of a particular plasmid, part of which plasmid is homologous to the T.reesei strain and contains DNA from the cbh1 locus with the entire cbh1 coding sequence removed therefrom, and replaced with a T.reesei gene which acts as a selectable marker for transformation.

In another preferred embodiment, the present invention is directed towards the preparation of a particular plasmid, part of which plasmid is homologous to the T. reesei strain and contains the cbh2 gene from the T.reesei strain with almost the entire cbh2 coding sequence removed therefrom and replaced with a T.reesei gene which acts as a selectable marker for transformation.

In another preferred embodiment, the present invention is directed towards the preparation of a particular plasmid part of which plasmid is homologous to the T. reesei strain and contains the egl3 gene from the T.reesei strain with the egl3 coding sequence disrupted by insertion of a T.reesei gene which acts as a selectable marker for transformation. The egl3 locus codes for the EGII protein.

In another preferred embodiment, the present invention is directed towards the preparation of a particular plasmid part of which plasmid is homologous to the T. reesei strain and contains the egl1 gene from the T.reesei strain with part of the egl1 coding sequence removed therefrom and replaced with a T.reesei gene which acts as a selectable marker for transformation.

In another preferred embodiment, the present invention is directed towards the preparation of a particular plasmid part of which contains DNA from the cbh1 locus with the entire cbh1 coding sequence removed therefrom and replaced with the egl1 gene from T. reesei and a T. reesei gene which acts as a selectable marker for transformation.

In another preferred embodiment, the present invention is directed towards the preparation of a particular plasmid part of which plasmid is homologous to the T. reesei strain and contains a xylanase gene from the T. reesei strain and a T. reesei gene which acts as a selectable marker for transformation.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is an outline of the construction of pΔCBHIpyr4.

FIG. 2 illustrates deletion of the T. reesei gene by integration of the larger EcoRI fragment from pΔCBHIpyr4 at the cbh1 locus on one of the T. reesei chromosomes.

FIG. 3 is an autoradiograph of DNA from T. reesei strain GC69 transformed with EcoRI digested pΔCBHIpyr4 after Southern blot analysis using a ^{32}P labelled pΔCBHIpyr4 as the probe. The sizes of molecular weight markers are shown in kilobase pairs to the left of the Figure.

FIG. 4 is an autoradiograph of DNA from a T. reesei strain GC69 transformed with EcoRI digested pΔCBHIpyr4 using a ^{32}P labelled pIntCBHI as the probe. The sizes of molecular weight markers are shown in kilobase pairs to the left of the Figure.

FIG. 5 is an isoelectric focusing gel displaying the proteins secreted by the wild type and by transformed strains of T. reesei. Specifically, in FIG.5, Lane A of the isoelectric focusing gel employs partially purified CBHI from T. reesei; Lane B employs a wild type T. reesei : Lane C employs protein from a T. reesei strain with the cbh1 gene deleted; and Lane D employs protein from a T. reesei strain with the cbh1 and cbh2 genes deleted. In FIG. 5, the right hand side of the figure is marked to indicate the location of the single proteins found in one or more of the secreted proteins. Specifically, BG refers to the β-glucosidase, E1 refers to endoglucanase I, E2 refers to endoglucanase II, E3 refers to endoglucanase III, C1 refers to exo-cellobiohydrolase I and C2 refers to exo-cellobiohydrolase II.

FIG. 6A is a representation of the T. reesei cbh2 locus, cloned as a 4.1 kb EcoRI fragment on genomic DNA and FIG. 6B is a representation of the cbh2 gene deletion vector pPΔCBHII.

FIG. 7 is an autoradiograph of DNA from T. reesei strain P37PΔCBHIPyr⁻26 transformed with EcoRI digested pPΔCBHII after Southern blot analysis using a ^{32}P labelled pPΔCBHII as the probe. The sizes of molecular weight markers are shown in kilobase pairs to the left of the Figure.

FIG. 8 is a diagram of the plasmid pEGIpyr4.

FIG. 9 is a diagram of the site specific alterations made in the egl1 and cbh1 genes to create convenient restriction endonuclease cleavage sites. In each case, the upper line shows the original DNA sequence, the changes introduced are shown in the middle line, and the new sequence is shown in the lower line.

FIG. 10 is a diagram of the larger EcoRI fragment which can be obtained from pCEPC1.

FIG. 11 is an autoradiograph of DNA, from an untransformed strain of T. reesei RutC30 and from two transformants obtained by transforming T. reesei with EcoRI digested pCEPC1. The DNA was digested with PstI, a Southern blot was obtained and hybridized with ^{32}P labelled pUC4K::cbh1. The sizes of marker DNA fragments are shown in kilobase pairs to the left of the Figure.

FIG. 12 is a diagram of the plasmid pEGII::P-1.

FIG 13. is an autoradiograph of DNA from T. reesei strain P37PΔΔ67P⁻1 transformed with HindIII and BamHI digested pEGII::P-1.

A Southern blot was prepared and the DNA was hybridized with an approximately 4kb PstI fragment of radiolabelled T.reesei DNA containing the egl3 gene. Lanes A, C and E contain DNA from the untransformed strain whereas, Lanes B, D and F contain DNA from the untransformed T. reesei strain. The T.reesei DNA was digested with BglII in Lanes A and B, with EcoRV in Lanes C and D and with PstI in Lanes E and F. The size of marker DNA fragments are shown in kilobase pairs to the left of the Figure.

FIG. 14 is a diagram of the plasmid pPΔEGI-1.

FIG. 15 is an autoradiograph of a Southern blot of DNA isolated from transformants of strain GC69 obtained with HindIII digested pΔEGIpyr-3. The pattern of hybridisation with the probe, radiolabelled pΔEGIpyr-3, expected for an untransformed strain is shown in Lane C. Lane A shows the pattern expected for a transformant in which the egl1 gene has been disrupted and Lane B shows a transformant in which pΔEGIpyr-3 DNA has integrated into the genome but without disrupting the egl1 gene. Lane D contains pΔEGIpyr-3 digested with HindIII to provide appropriate size markers. The sizes of marker DNA fragments are shown in kilobase pairs to the right of the figure.

FIG. 16 shows alignment of the deduced amino acid sequence of the cloned T. reesei genes with the sequence of other microbial xylanases. "High pI" indicates the sequence of the high pI xylanase of T. reesei, "Low pI" the sequence of Low pI xylanase of T. reesei, "trichv" the sequence of a T. viride xylanase disclosed by M. Yaguchi, Institute of Biological Sciences, National Research Council of Canada at

the Fourth Chemical Congress of North America, New York, August 25-30, 1991, and "baccir" the sequence of Bicillus circulans xylanase, "bacpum" the sequence of Bacillus pumilus xylanase.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

As used herein, the term "homologous DNA" means that the DNA contains no DNA sequences from a microorganism other than T. reesei.

The term "substantially homologous recombinant DNA" means the recombinant DNA is derived from T. reesei or is synthesized to conform to the DNA sequence in T. reesei and contains no more than 50 base pairs of contiguous synthetic DNA. More preferably, the recombinant DNA is derived from T.reesei or is synthesized to conform to the DNA sequence of T.reesei and contains no more than 25 base pairs of contiguous synthetic DNA. According to current guidelines "incorporation of fully sequenced DNA of 25 base pairs or less is not considered to comprise modifications to host vector systems." (U.S. Department of Health, Education, and Welfare, Public Health Service, National Institute of Health. Modification of Certified Host-Vector Systems. Recombinant-DNA Technical Bulletin 2 (3): 132, 1979).

The term "heterologous DNA" means any source of DNA that is nonsynthetically produced from a microorganism other than T. reesei or any piece of synthetic DNA greater than 50 base pairs not synthesized

to conform to the DNA sequence of T. reesei. "Heterologous protein" means protein encoded by heterologous DNA.

The term "homologous recombination" means that the recombinant DNA integrated at a specific location within the genome which has the same DNA sequence as part of the recombinant DNA and did not integrate at secondary sites.

The term "Endoglucanase ("EG") components" refer to all of those fungal cellulase components or combination of components which are the endoglucanase components of T. reesei (specifically , EGI, EGII, EGIII, and the like, either alone or in combination).

The term "exo-cellobiohydrolase ("CBH") components" refer to those fungal cellulase components which are the exo-cellobiohydrolase components of T. reesei (specifically CBHI, CBHII and the like, either alone or in combination).

The term " cells" means both the cells and the protoplasts created from the cells of T. reesei.

The term "overexpress" means that an additional copy of a gene has been integrated into the genome so that when the protein encoded by the gene is expressed, the protein is produced at quantities greater than if only one copy of the gene was present in the genome.

The present invention relates to the precise replacement of chromosomal regions with DNA sequences that may or may not be

altered in vitro by using recombinant DNA techniques. Total gene replacement in the transformed host microorganism is possible. It is further contemplated by the present invention, that the native cbh1, cbh2, egl1, or egl3 genes, or any other cloned T. reesei gene can be altered, such as a deletion or deletions of specific nucleic acids within the gene by techniques known in this art and used to replace the natural gene in the transformed microorganism. For example, amino acids that are present at the catalytic site of the protein may be deleted or substituted with different amino acids. These in vitro alterations may produce cellulase proteins that have altered specific activity with certain substrates, altered end product inhibition, altered sensitivity to oxidation and/or altered temperature or pH activity profiles for the enzyme.

Also contemplated by the present invention is manipulation of the T. reesei strain via transformation such that certain targeted genes are deleted or disrupted within the genome and extra copies of certain native genes such as egl1, egl3 and the like can be homologously recombined into the strain. Since T. reesei, is a mesophilic, saprophytic filamentous fungus which secretes different cellulolytic enzymes, the transformants can be used to produce the desired cellulase enzyme or combination of enzymes thereof. However, the present invention is not limited to gene manipulation of only cellulolytic enzymes. Any alteration of any gene in the fungus T. reesei is contemplated by the present invention and any T. reesei gene which has been cloned can be deleted from the genome or be disrupted, including, but not limited to cbh1, cbh2, egl1, egl3, genes encoding other endoglucanases, ß-glucosidase or xylanases or other

carbohydrases, genes required for uridine biosynthesis (eg. pyr4) arginine biosynthesis, tryptophan biosynthesis and the like. Multiple deletions are also possible, such as deletions of both the cbh1 and cbh2 genes both the egl1 and egl3 genes, and of the cbh1, cbh2, egl1 and egl3 genes.

A selectable marker must first be chosen so as to enable detection of the transformed fungus. Any selectable marker gene which is naturally present in T. reesei, can be used in the present invention so that its presence in the transformants will not materially affect the properties thereof. The selectable marker can be a gene which encodes an assayable product. The selectable marker may be a functional copy of a T. reesei gene which, if lacking in the host strain results in the host strain displaying an auxotrophic phenotype. The selectable marker may be derived from a T. reesei gene which specifies a novel phenotype such as an ability to utilize a metabolite that is usually not metabolized by T. reesei or the ability to resist toxic effects of a chemical or demonstrate resistance to an antibiotic. Also contemplated within the present invention are synthetic gene markers that can be synthesized by methods known in the art. These synthetic genes should contain DNA sequences that mimic the gene sequences in T. reesei. Transformants can then be selected on the basis of the selectable marker introduced therein.

The host strains used could be derivatives of T. reesei which lack or have a nonfunctional gene or genes corresponding to the selectable marker chosen. For example, if the selectable marker of pyr4 is used, then a specific pyr⁻ derivative strain is used as a recipient

in the transformation procedure. Other examples of selectable markers that can be used in the present invention include the T. reesei genes equivalent to the Aspergillus nidulans genes argB, trpC, niaD, and the like. The corresponding recipient strain must therefore be a derivative strain such as argB⁻, TrpC⁻, niaD⁻, and the like.

The strain is derived from a starting host strain which is any T. reesei strain. However it is preferable to use a T. reesei over-producing strain such as RL-P37, described by Sheir-Neiss et al. in Appl. Microbiol. Biotechnology, 20 (1984) pp. 46-53, since this strain secretes elevated amounts of proteins and in particular elevated amounts of cellulase enzymes. This strain is then used to produce the derivative strains used in the transformation process.

The derivative strain of T. reesei can be prepared by a number of techniques known in the art such as the filtration enrichment technique described by Nevalainen which is incorporated herein by reference (Nevalainen, 1985). Another technique to obtain the derivative strain is to identify the derivatives under different growth medium conditions. For instance, the argB- derivatives can be identified by using a series of minimal plates supplied by different intermediates in arginine biosynthesis. Another example is the production of pyr4- derivative strains by subjecting the strains to fluoroorotic acid (FOA). The pyr4 gene encodes orotidine-5′-monophosphate decarboxylase, an enzyme required for the biosynthesis of uridine. Strains with an intact pyr4 gene grow in a medium lacking uridine but are sensitive to fluoroorotic acid. It is possible to select pyr4- derivative strains which lack a functional orotidine monophosphate decarboxylase enzyme and require

uridine for growth by selecting for FOA resistance. Using the FOA selection technique it is also possible to obtain uridine requiring strains which lack a functional orotate pyrophosphoribosyl transferase. It is possible to transform these cells with a functional copy of the gene encoding this enzyme (Berges and Barreau, 1991, Curr. Genet. 19 pp359-365). Since it is easy to select derivative strains using the FOA resistance technique in the present invention, it is preferable to use the pyr4 gene as a selectable marker.

Any plasmid can be used in the present invention for the cloning of the selectable marker such as pUC-derivatives, pBR322 and the like. The plasmid used is chosen on the basis of the convenience of restriction enzyme sites that permit the incorporation of the selectable marker into the plasmid with ease. In the present invention, it is preferable to use the plasmid pUC18, which contains a single HindIII restriction site.

The selectable marker is then cloned into the respective plasmid using techniques known in the art, which techniques are set forth in Maniatis et al. (1989), and is incorporated herein by reference. The pyr4 gene of T. reesei can be cloned into the pUC18 plasmid by the methods described by Smith et al. (1991).

A region of the T. reesei genome which encompasses the coding sequence of the gene to be deleted from the T. reesei strain through transformation is then cloned into a second plasmid by methods known in the art. Any gene from the strain T. reesei which has been cloned can be deleted such as cbh1, cbh2, egl1, egl3 and the like. In addition

to deleting genes from the genome of the transformants, the addition of extra copies of a gene is also possible. For instance, a transformant may be desired that has extra copies of the egl1 gene. The present invention encompasses methods to also add these additional copies of the gene or genes.

The plasmid for gene deletion and/or addition is selected such that restriction enzyme sites are present therein to enable the fragment of homologous DNA to be removed as a single linear piece. For example, it is preferable to use a pUC4K plasmid for deletion of cbh1 because it has symmetrical EcoRI and PstI restriction sites in a polylinker region.

The desired gene that is to be deleted from the transformant is inserted into the plasmid by methods known in the art. The plasmid containing the gene to be deleted or disrupted is then cut at the appropriate restriction enzyme site(s), the gene coding sequence or part thereof may be removed therefrom and the selectable marker inserted. Flanking DNA sequences from the locus of the gene to be deleted or disrupted, preferably between about 0.5 to 2.0 kb, remain on either side of the selectable marker gene. If the flanking region is too small, then homologous integration occurs infrequently during transformation.

A preferred embodiment for preparing appropriate plasmid vectors utilizes the E. coli vector plasmids pUC4K and pUC18. The pUC4K plasmid vector has the cbh1 gene which was originally obtained from genomic DNA of the T. reesei strain RL-P37 by

hybridization with an appropriate oligonucleotide probe designed on the basis of the published sequence for the cbh1 gene. The cbh1 gene was inserted into the pUC4K vector by cutting the vector with PstI, resulting in the removal of the Kan^r gene therefrom and ligating with a PstI fragment of T. reesei DNA containing the cbh1 gene. The resulting plasmid, pUC4K::cbhI was cut with HindIII and the larger fragment of about 6 kb was isolated and religated to produce plasmid pUC4K::cbhIΔH/H. This procedure removed the entire cbh1 coding sequence and approximately 1.2 kb upstream and 1.5 kb downstream flanking sequences. Approximately 1 kb of flanking DNA from either end of the original PstI fragment remains.

The plasmid pUC4K::cbhIΔH/H was cut with HindIII and the ends were dephosphorylated with calf intestinal alkaline phosphatase to prevent self-ligation of the vector. This DNA was then ligated with a 6.5 kb HindIII pyr4 gene fragment to create pΔCBHIpyr4. A much smaller fragment of DNA bearing the pyr4 gene also can be used.

Another preferred embodiment for preparing appropriate plasmid vectors in the present invention is diagrammatically illustrated in FIG. 6A. The cbh2 gene of T. reesei, encoding the CBHII protein, has been cloned as a 4.1 kb EcoRI fragment of genomic DNA (Chen et al., 1987). Using methods known in the art, the plasmid pPΔCBHII has been constructed in which a 1.7 kb central region of the cbh2 gene between a HindIII site and a ClaI site has been removed and replaced with the T. reesei pyr4 gene.

In another preferred embodiment, a plasmid has been constructed that contains the T. reesei pyr4 and egl1 genes joined end to end. Isolation of the linear fragment containing the T. reesei genes and transformation of a pyr4⁻ strain should allow multiple copies of the egl1 gene to be integrated into the genome without any plasmid integration. This plasmid is illustrated diagrammatically in FIG. 8.

A plasmid, pCEPC1, also has been constructed in which the promotor from the cbh1 gene has been fused to the coding sequence of the egl1 gene, while maintaining the egl1 terminator region. The 3' flanking region of the cbh1 locus follows the egl1 terminator region. The pyr4 gene is inserted into the 3' flanking region of the cbh1 locus.

Another preferred embodiment for preparing appropriate plasmid vectors in the present invention is diagrammatically illustrated in FIG. 12. The egl3 gene of T. reesei, encoding the EGII protein, has been cloned as a 4 kb PstI-XhoI fragment of genomic DNA (Saloheimo et al., 1988, Gene 63, p.11-21). The plasmid pEGII::P-1 has been constructed in which a 2.7 kb SalI fragment containing theT. reesei pyr4 gene was inserted into a SalI site within the EGII coding sequence resulting in disruption of the EGII coding sequence.

Another preferred embodiment for preparing appropriate plasmid vectors in the present invention is diagrammatically illustrated in FIG. 14. The egl1 gene of T. reesei, encoding the EGI protein, has been cloned as a 4.2 kb HindIII fragment of genomic DNA (Pentilla et al., 1986, Gene 45, pp. 253-263; van Arsdell et al., 1987, BioTechnology

5, pp. 60-64). The plasmid pPΔEGI-1 has been constructed in which a 1 kb region from the center of the EGI coding sequence to a position beyond the 3' end of the coding sequence was removed and replaced with the T. reesei pyr4 gene.

The specific plasmids were linearized with restriction enzymes to produce an homologous DNA fragment containing the selectable marker. The marker is preferably between two flanking regions which act to integrate the selectable marker at a precise locus in the derivative T. reesei strain during the transformation process. Although the transforming DNA may sometimes integrate into secondary sites, transformants in which only a single copy of the linear DNA integrated into the desired locus can be identified by methods described in the specific examples given below.

Although specific plasmid vectors are described above, the present invention is not limited to the production of these vectors. Various genes can be deleted and replaced in the T. reesei strain using the above techniques. Any available selectable markers can be used, as discussed below. Potentially any T. reesei gene which has been cloned, and thus identified, can be deleted from the genome using the above-described strategy. For instance, the cbh1, cbh2, egl1 and egl3 genes can be deleted and replaced by a selectable marker gene. All of these variations are included within the present invention.

Since the permeability of the cell wall in T. reesei is very low, uptake of the desired DNA sequence, gene or gene fragment is at best minimal. There are a number of methods to increase the permeability

of the T. reesei cell wall in the derivative strain (i.e., lacking a functional gene corresponding to the used selectable marker) prior to the transformation process.

One method that may be used involves the addition of alkali metal ions and/or alkaline earth metal ions to a high concentration to T. reesei cells. Any alkali metal or alkaline earth metal may be used in the present invention, however it is preferable to use either $CaCl_2$ or lithium acetate and more preferable to use lithium acetate. The concentration of the alkali metal or alkaline earth metal may vary depending on the ion used. Generally between about 0.05 M to 0.4 M concentrations of alkali metal ions are used. It is preferable to use about a 0.1 M concentration of alkali earth metals. Preferably the lithium acetate concentration is about 0.1 M.

Another method that can be used to induce cell wall permeability to enhance DNA uptake in T. reesei is to resuspend the cells in a growth medium supplemented with sorbitol and carrier calf thymus DNA. Glass beads are then added to the supplemented medium and the mixture is vortexed at high speed for about 30 seconds. This treatment disrupts the cell walls, but may kill many of the cells.

Yet another method to prepare T. reesei for transformation involves the preparation of protoplasts from fungal mycelium. The mycelium can be obtained from germinated vegetative spores. The mycelium is treated with an enzyme which digests the cell wall resulting in protoplasts. The protoplasts are then protected by the presence of an osmotic stabilizer in the suspending medium. These

stabilizers include sorbitol, mannitol, potassium chloride, magnesium sulfate and the like. Usually the concentration of these stabilizers varies between 0.8 M to 1.2 M. It is preferabie to use about a 1.2 M solution of sorbitol in the suspension medium.

Uptake of the DNA into the host T. reesei strain is dependent upon the calcium ion concentration. Generally between about 10 mM $CaCl_2$ and 50 mM $CaCl_2$ is used in an uptake solution. Besides the need for the calcium ion in the uptake solution, other items generally included are a buffering system such as TE buffer (10 mM Tris, pH 7.4; 1 mM EDTA) or 10 mM MOPS, pH 6.0 buffer (morpholinepropanesulfonic acid) and polyethylene glycol (PEG). It is believed that the polyethylene glycol acts to fuse the cell membranes thus permitting the contents of the medium to be delivered into the cytoplasm of the T. reesei strain and the plasmid DNA is transferred to the nucleus. This fusion frequently leaves multiple copies of the plasmid DNA tandemly integrated into the host chromosome.

Usually a suspension containing the T. reesei protoplasts or cells that have been subjected to a permeability treatment at a density of 10^8 to 10^9/ml, preferably 2 x 10^8/ml are used in transformation. These protoplasts or cells are added to the uptake solution, along with the desired linearized selectable marker having substantially homologous flanking regions on either side of said marker to form a transformation mixture. Generally a high concentration of PEG is added to the uptake solution. From 0.1 to 1 volume of 25% PEG 4000 can be added to the protoplast suspension. However, it is preferable to add about 0.25 volumes to the protoplast suspension. Additives such as dimethyl

sulfoxide, heparin, spermidine, potassium chloride and the like may also be added to the uptake solution and aid in transformation.

Generally, the mixture is then incubated at approximately 0°C for a period between 10 to 30 minutes. Additional PEG is then added to the mixture to further enhance the uptake of the desired gene or DNA sequence. The 25% PEG 4000 is generally added in volumes of 5 to 15 times the volume of the transformation mixture; however, greater and lesser volumes may be suitable. The 25% PEG 4000 is preferably about 10 times the volume of the transformation mixture. After the PEG is added, the transformation mixture is then incubated at room temperature before the addition of a sorbitol and $CaCl_2$ solution. The protoplast suspension is then further added to molten aliquots of a growth medium. This growth medium permits the growth of transformants only. Any growth medium can be used in the present invention that is suitable to grow the desired transformants. However, if Pyr$^+$ transformants are being selected it is preferable to use a growth medium that contains no uridine. The subsequent colonies are transferred and purified on a growth medium depleted of uridine.

At this stage, stable transformants were distinquished from unstable transformants by their faster growth rate and the formation of circular colonies with a smooth, rather than ragged outline on solid culture medium lacking uridine. Additionally, in some cases a further test of stability was made by growing the transformants on solid non-selective medium (i.e. containing uridine), harvesting spores from this culture medium and determining the percentage of these spores which

will subsequently germinate and grow on selective medium lacking uridine.

In one preferred embodiment the transformant produced by using the linear DNA fragment from pΔCBHIpyr4 is strain P37PΔCBHI. This strain has the cbh1 gene deleted. FIG. 2 illustrates diagrammatically a deletion of the T. reesei cbh1 gene by integration of the larger EcoRI fragment from pΔCBHIpyr4 at the cbh1 locus on one of the T. reesei chromosomes. In another preferred embodiment, the linear DNA fragment from pΔCBHIpyr4 can be used to transform a T. reesei strain in which other cellulase component genes have been deleted or overexpressed in order to create a transformant in which at least the cbh1 gene has been deleted.

In another preferred embodiment, a linearized substantially homologous DNA fragment can be prepared containing flanking DNA sequences from the T. reesei cbh2 locus located on either side of the T. reesei pyr4 gene. For example, transformation of GC69, a pyr4⁻ derivative, with the linear fragment will result in a transformant having the cbh2 gene deleted. Similarly, transformation of a pyr4⁻ derivative of P37PΔCBHI with the linear fragment and selection for growth on medium lacking uridine will result in a transformant having both the cbh1 and cbh2 genes deleted. In another preferred embodiment, the linear DNA fragment can be used to transform a T. reesei strain in which other cellulase component genes have been deleted or overexpressed in order to create a transformant in which at least the cbh2 gene has been deleted.

In another preferred embodiment, a linearized substantially homologous DNA fragment can be prepared encoding the egl1 locus with a part of the coding sequence replaced with the T. reesei pyr4 gene. For example, transformation of GC69, with the linear DNA fragment will result in a transformant having the egl1 gene deleted. In another preferred embodiment, the linear DNA fragment can be used to transform a T. reesei strain in which other cellulase component genes have been deleted or overexpressed in order to create a transformant in which at least the egl1 gene has been deleted. Such transformants will be unable to produce the EGI component of cellulase derived from T. reesei.

In another preferred embodiment, a linearized substantially homologous DNA fragment can be prepared encoding the egl3 locus with the egl3 coding sequence disrupted by the insertion of the T. reesei pyr4 gene. For example, transformation of GC69, with the linear fragment will result in a transformant having the egl3 gene deleted. In another preferred embodiment, the linear DNA fragment can be used to transform a T. reesei pyr$^-$ strain in which other cellulase component genes have been deleted or overexpressed in order to create a transformant in which at least the egl3 gene has been deleted. Such transformants will be unable to produce the EGII component of cellulase derived from T. reesei.

In another embodiment, a linearized substantially homologous DNA fragment containing a promotor from the cbh1 gene can be fused to the coding sequence of an egl1 gene. The pyr4$^-$ gene and the 3' flanking region from the cbh1 are then ligated to the fragment. For

example, transformation of a T. reesei pyr4⁻ strain with a linear fragment from pCEPC1 containing the egl1 gene and selection for growth in the absence of uridine should result in a transformant containing a copy of the egl1 gene under the control of the cbh1 promotor at the cbh1 locus, in addition to the native egl1 gene. In another preferred embodiment, the linear DNA fragment from pCEPC1 can be used to transform a T. reesei pyr⁻ strain in which other cellulase component genes have been deleted or overexpressed in order to create a transformant in which a number of cellulase components have been deleted and in which at least the egl1 gene is being overexpressed.

In another preferred embodiment, a linearized substantially homologous DNA fragment containing either the T.reesei low pI or high pI xylanase gene and a T.reesei selectable marker can be prepared. Transformation of T.reesei cells with this DNA fragment should result in transformants which overexpress a xylanase protein.

In order to ensure that the transformation occurred by the above-described methods, further analysis can be performed on the transformants such as autoradiography of Southern blots, and isoelectric focusing of secreted proteins.

After confirmation that the transformed strains lack a specific gene or genes or contain extra gene copies and that they contain no foreign DNA, the transformants are then further cultured. The secreted proteins from the transformed culture can then be obtained and used in

a cellulase composition, which composition lacks the deleted proteins and/or contains the enhanced proteins.

The microorganisms modified in the above manner are particularly useful in preparing cellulase compositions having one or more deleted components. In turn, such cellulase compositions impart improved properties per specific application as compared to cellulases containing naturally occurring ratios of EG components to CBH components. In particular, it has been found that cellulase compositions deficient in CBHI components, and preferably deficient in CBHI and CBHII components, are useful in detergent cleaning compositions, e.g., laundry detergent compositions, and provide for improved color restoration, softening, etc. while providing reduced strength loss to cotton-containing fabrics. See, for instance U.S. Patent Application Serial No. 07/713,738 which is incorporated herein by reference in its entirety. Additionally, when such EG enriched cellulase compositions contain some CBHI components (but less than 5 weight percent based on the total weight of the cellulase composition), then such cellulase compositions also impart cleaning. Even more suprising is the fact that CBHII cellulase components do not substitute for CBHI cellulase components (at the levels tested) in providing cleaning benefits when combined with EG-type components in detergent compositions.

It is also noted that CBHI enriched cellulase compositions (i.e, having a ratio of CBHI to all EG components of greater than 5:1) as well as EG compositions containing less than about 5 weight percent of CBHI components, impart degradation resistance to the detergent

composition as compared to detergent compositions containing whole cellulase systems. See, for example, U.S. Patent Application Serial No. 07/422,814, filed October 19, 1989, and U.S. Patent Application Serial No. 07/713,738 which are incorporated herein by reference in their entirety. That is to say that cotton fabrics treated with such cellulase compositions provide for less strength loss when treated over repeated washings as compared to the strength loss resulting from whole cellulase systems. As is apparent, such cellulase compositions enriched or deficient in the CBHI component can be produced by selectively altering the ability of the microorganism to produce one or more of the cellulase components.

In a preferred embodiment, the EG cellulase having less than about 5 weight percent of CBHI component described herein can be prepared by modifying T. reesei in the manner described above so that this microorganism is unable to produce CBHI and preferably CBHI and CBHII components. The modified microorganisms of this invention are particularly suitable for preparing such compositions because they produce cellulase compositions which lack all of the CBH components whereas prior art purification techniques cannot.

In another embodiment, it has also been found that the EGIII component of T. reesei is useful in detergent compositions and, because of its high activity at pH 7 - 8, is particularly suited for use in neutral/alkaline detergent compositions. See, for example, U.S. Patent Application Serial No. 07/747,647 which is incorporated herein by reference. One method for preparing a cellulase compostion enriched in EGIII is to delete CBHI, CBHII, EGI and EGII.

In regard to the detergent compositions containing cellulase compostions which are CBHI deficient, CBHI enriched or EGIII enriched, it has been found that it is the amount of cellulase, and not the relative rate of hydrolysis of the specific enzymatic components to produce reducing sugars from cellulose, which imparts the desired detergent properties to cotton-containing fabrics, eg., one or more of improved color restoration, improved softening and improved cleaning to the detergent composition.

The CBHI deficient cellulase compositions are also useful in improving the feel and appearance of cotton fabrics and garments ("cotton fabrics" - 100% cotton and blends having up to 40% cotton) by treating the fabrics with a solution containing a cellulase solution deficient in CBHI and preferably CBHI and CBHII. In this regard, the cellulase compositions not only improve the appearance of the cotton fabric but also impart improved softening and degradation resistance to the fabric as compared to whole cellulase compositions (systems).

Such methods are particularly suited for textile applications as disclosed in U.S. Patent Application Serial No. 07/677,385 and U.S. Patent Application Serial No. 07/678,865, both of which are incorporated herein by reference in their entirety. In such embodiments, the cellulase composition has a ratio of all EG components to all CBHI components of 5:1 and greater and is preferably free of CBHI components and more preferably free of all CBH components. As is apparent, such cellulase compositions could be prepared by the methods described herein by the selective deletion of cellulase genes from T. reesei.

In order to further illustrate the present invention and advantages thereof, the following specific examples are given, it being understood that the same are intended only as illustrative and in nowise limitative.

EXAMPLES

Example 1

Selection for pyr4⁻ derivatives of Trichoderma reesei

The pyr4 gene encodes orotidine-5'-monophosphate decarboxylase, an enzyme required for the biosynthesis of uridine. The toxic inhibitor 5-fluoroorotic acid (FOA) is incorporated into uridine by wild-type cells and thus poisons the cells. However, cells defective in the pyr4 gene are resistant to this inhibitor but require uridine for growth. It is, therefore, possible to select for pyr4 derivative strains using FOA. In practice, spores of T. reesei strain RL-P37 (Sheir-Neiss, G. and Montenecourt, B.S., Appl. Microbiol. Biotechnol. 20, p. 46-53 (1984)) were spread on the surface of a solidified medium containing 2 mg/ml uridine and 1.2 mg/ml FOA. Spontaneous FOA-resistant colonies appeared within three to four days and it was possible to subsequently identify those FOA-resistant derivatives which required uridine for growth. In order to identify those derivatives which specifically had a defective pyr4 gene, protoplasts were generated and transformed with a plasmid containing a wild-type pyr4 gene (see Examples 3 and 4). Following transformation, protoplasts were plated on medium lacking uridine. Subsequent growth of transformed colonies demonstrated complementation of a defective pyr4 gene by the plasmid-borne pyr4 gene. In this way, strain GC69 was identified as a pyr4⁻ derivative of strain RL-P37.

Example 2

Preparation of CBHI Deletion Vector

A cbh1 gene encoding the CBHI protein was cloned from the genomic DNA of T. reesei strain RL-P37 by hybridization with an oligonucleotide probe designed on the basis of the published sequence for this gene using known probe synthesis methods (Shoemaker et al., 1983b). The cbh1 gene resides on a 6.5 kb PstI fragment and was inserted into PstI cut pUC4K (purchased from Pharmacia Inc., Piscataway, NJ) replacing the Kan^r gene of this vector using techniques known in the art, which techniques are set forth in Maniatis et al., (1989) and incorporated herein by reference. The resulting plasmid, pUC4K::cbh1 was then cut with HindIII and the larger fragment of about 6 kb was isolated and religated to give pUC4K::cbh1ΔH/H (see FIG. 1). This procedure removes the entire cbh1 coding sequence and approximately 1.2 kb upstream and 1.5 kb downstream of flanking sequences. Approximately, 1 kb of flanking DNA from either end of the original PstI fragment remains.

The T. reesei pyr4 gene was cloned as a 6.5 kb HindIII fragment of genomic DNA in pUC18 to form pTpyr2 (Smith et al., 1991) following the methods of Maniatis et al., supra. The plasmid pUC4K::cbhIΔH/H was cut with HindIII and the ends were dephosphorylated with calf intestinal alkaline phosphatase. This end dephosphorylated DNA was ligated with the 6.5 kb HindIII fragment containing the T. reesei pyr4 gene to give pΔCBHIpyr4. FIG. 1 illustrates the construction of this plasmid.

Example 3

Isolation of Protoplasts

Mycelium was obtained by inoculating 100 ml of YEG (0.5% yeast extract, 2% glucose) in a 500 ml flask with about 5×10^7 T. reesei GC69 spores (the pyr4⁻ derivative strain). The flask was then incubated at 37°C with shaking for about 16 hours. The mycelium was harvested by centrifugation at 2,750 x g. The harvested mycelium was further washed in a 1.2 M sorbitol solution and resuspended in 40 ml of a solution containing 5 mg/ml Novozym^R 234 solution (which is the tradename for a multicomponent enzyme system containing 1,3-alpha-glucanase, 1,3-beta-glucanase, laminarinase, xylanase, chitinase and protease from Novo Biolabs, Danbury, Ct.); 5 mg/ml $MgSO_4.7H_2O$; 0.5 mg/ml bovine serum albumin; 1.2 M sorbitol. The protoplasts were removed from the cellular debris by filtration through Miracloth (Calbiochem Corp, La Jolla, CA) and collected by centrifugation at 2,000 x g. The protoplasts were washed three times in 1.2 M sorbitol and once in 1.2 M sorbitol, 50 mM $CaCl_2$, centrifuged and resuspended at a density of approximately 2×10^8 protoplasts per ml of 1.2 M sorbitol, 50 mM $CaCl_2$.

Example 4

Transformation of Fungal Protoplasts with pΔCBHIpyr4

200 µl of the protoplast suspension prepared in Example 3 was added to 20 µl of EcoRI digested pΔCBHIpyr4 (prepared in Example 2) in TE buffer (10 mM Tris, pH 7.4; 1 mM EDTA) and 50 µl of a

polyethylene glycol (PEG) solution containing 25% PEG 4000, 0.6 M KCl and 50 mM $CaCl_2$. This mixture was incubated on ice for 20 minutes. After this incubation period 2.0 ml of the above-identified PEG solution was added thereto, the solution was further mixed and incubated at room temperature for 5 minutes. After this second incubation, 4.0 ml of a solution containing 1.2 M sorbitol and 50 mM $CaCl_2$ was added thereto and this solution was further mixed. The protoplast solution was then immediately added to molten aliquots of Vogel's Medium N (3 grams sodium citrate, 5 grams KH_2PO_4, 2 grams NH_4NO_3, 0.2 grams $MgSO_4.7H_2O$, 0.1 gram $CaCl_2.2H_2O$, 5 μg σ-biotin, 5 mg citric acid, 5 mg $ZnSO_4.7H_2O$, 1 mg $Fe(NH_4)_2.6H_2O$, 0.25 mg $CuSO_4.5H_2O$, 50 μg MnSO4.4H20 per liter) containing an additional 1% glucose, 1.2 M sorbitol and 1% agarose. The protoplast/medium mixture was then poured onto a solid medium containing the same Vogel's medium as stated above. No uridine was present in the medium and therefore only transformed colonies were able to grow as a result of complementation of the pyr4 mutation of strain GC69 by the wild type pyr4 gene insert in pΔCBHIpyr4. These colonies were subsequently transferred and purified on a solid Vogel's medium N containing as an additive, 1% glucose and stable transformants were chosen for further analysis.

At this stage stable transformants were distinquished from unstable transformants by their faster growth rate and formation of circular colonies with a smooth, rather than ragged outline on solid culture medium lacking uridine. In some cases a further test of stability was made by growing the transformants on solid non-selective medium (i.e. containing uridine), harvesting spores from this medium

and determining the percentage of these spores which will subsequently germinate and grow on selective medium lacking uridine.

Example 5

Analysis of the Transformants

DNA was isolated from the transformants obtained in Example 4 after they were grown in liquid Vogel's medium N containing 1% glucose. These transformant DNA samples were further cut with a PstI restriction enzyme and subjected to agarose gel electrophoresis. The gel was then blotted onto a Nytran membrane filter and hybridized with a ^{32}P labelled pΔCBHIpyr4 probe. The probe was selected to identify the native cbh1 gene as a 6.5 kb PstI fragment, the native pyr4 gene and any DNA sequences derived from the transforming DNA fragment.

The radioactive bands from the hybridization were visualized by autoradiography. The autoradiograph is seen in FIG. 3. Five samples were run as described above, hence samples A, B, C, D, and E. Lane E is the untransformed strain GC69 and was used as a control in the present analysis. Lanes A-D represent transformants obtained by the methods described above. The numbers on the side of the autoradiograph represent the sizes of molecular weight markers. As can be seen from this autoradiograph, lane D does not contain the 6.5 kb CBHI band, indicating that this gene has been totally deleted in the transformant by integration of the DNA fragment at the cbh1 gene. The cbh1 deleted strain is called P37PΔCBHI. Figure 2 outlines the deletion of the T. reesei cbh1 gene by integration through a double

cross-over event of the larger EcoRI fragment from pΔCBHIpyr4 at the cbh1 locus on one of the T. reesei chromosomes. The other transformants analyzed appear identical to the untransformed control strain.

Example 6

Analysis of the Transformants with pIntCBHI

The same procedure was used in this example as in Example 5, except that the probe used was changed to a ^{32}P labelled pIntCBHI probe. This probe is a pUC-type plasmid containing a 2 kb BglII fragment from the cbh1 locus within the region that was deleted in pUC4K::cbh1ΔH/H. Two samples were run in this example including a control, sample A, which is the untransformed strain GC69 and the transformant P37PΔCBHI, sample B. As can be seen in FIG. 4, sample A contained the cbh1 gene, as indicated by the band at 6.5 kb; however the transformant, sample B, does not contain this 6.5 kb band and therefore does not contain the cbh1 gene and does not contain any sequences derived from the pUC plasmid.

Example 7

Protein Secretion by Strain P37PΔCBHI

Spores from the produced P37PΔCBHI strain were inoculated into 50 ml of a Trichoderma basal medium containing 1% glucose, 0.14% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.03% $MgSO_4$, 0.03% urea, 0.75%

bactotryptone, 0.05% Tween 80, 0.000016% $CuSO_4.5H_2O$, 0.001% $FeSO_4.7H_2O$, 0.000128% $ZnSO_4.7H_2O$, 0.0000054% $Na_2MoO_4.2H_2O$, 0.0000007% MnCl.4H2O). The medium was incubated with shaking in a 250 ml flask at 37°C for about 48 hours. The resulting mycelium was collected by filtering through Miracloth (Calbiochem Corp.) and washed two or three times with 17 mM potassium phosphate. The mycelium was finally suspended in 17 mM potassium phosphate with 1 mM sophorose and further incubated for 24 hours at 30°C with shaking. The supernatant was then collected from these cultures and the mycelium was discarded. Samples of the culture supernatant were analyzed by isoelectric focusing using a Pharmacia Phastgel system and pH 3-9 precast gels according to the manufacturer's instructions. The gel was stained with silver stain to visualize the protein bands. The band corresponding to the cbh1 protein was absent from the sample derived from the strain P37PΔCBHI, as shown in FIG. 5. This isoelectric focusing gel shows various proteins in different supernatant cultures of T. reesei. Lane A is partially purified CBHI; Lane B is the supernatant from an untransformed T. reesei culture; Lane C is the supernatant from strain P37PΔCBHI produced according to the methods of the present invention. The position of various cellulase components are labelled CBHI, CBHII, EGI, EGII, and EGIII. Since CBHI constitutes 50% of the total extracellular protein, it is the major secreted protein and hence is the darkest band on the gel. This isoelectric focusing gel clearly shows depletion of the CBHI protein in the P37PΔCBHI strain.

Example 8

Preparation of pPΔCBHII

The cbh2 gene of T. reesei, encoding the CBHII protein, has been cloned as a 4.1 kb EcoRI fragment of genomic DNA which is shown diagramatically in FIG. 6A (Chen et al., 1987, Biotechnology, 5:274-278). This 4.1 kb fragment was inserted between the EcoRI sites of pUC4XL. The latter plasmid is a pUC derivative (constructed by R.M. Berka, Genencor International Inc.) which contains a multiple cloning site with a symetrical pattern of restriction endonuclease sites arranged in the order shown here: EcoRI, BamHI, SacI, SmaI, HindIII, XhoI, BglII, ClaI, BglII, XhoI, HindIII, SmaI, SacI, BamHI, EcoRI. Using methods known in the art, a plasmid, pPΔCBHII (FIG. 6B), has been constructed in which a 1.7 kb central region of this gene between a HindIII site (at 74 bp 3' of the CBHII translation initiation site) and a ClaI site (at 265 bp 3' of the last codon of CBHII) has been removed and replaced by a 1.6 kb HindIII- ClaI DNA fragment containing the T. reesei pyr4 gene.

The T. reesei pyr4 gene was excised from pTpyr2 (see Example 2) on a 1.6 kb NheI-SphI fragment and inserted between the SphI and XbaI sites of pUC219 (see Example 16) to create p219M (Smith et al., 1991, Curr. Genet 19 p. 27-33). The pyr4 gene was then removed as a HindIII-ClaI fragment having seven bp of DNA at one end and six bp of DNA at the other end derived from the pUC219 multiple cloning site and inserted into the HindIII and ClaI sites of the cbh2 gene to form the plasmid pPΔCBHII (see FIG. 6B).

Digestion of this plasmid with EcoRI will liberate a fragment having 0.7 kb of flanking DNA from the cbh2 locus at one end, 1.7 kb of flanking DNA from the cbh2 locus at the other end and the T. reesei pyr4 gene in the middle.

Example 9

Deletion of the cbh2 gene in T. reesei strain GC69

Protoplasts of strain GC69 will be generated and transformed with EcoRI digested pPΔCBHII according to the methods outlined in Examples 3 and 4. DNA from the transformants will be digested with EcoRI and Asp718, and subjected to agarose gel electrophoresis. The DNA from the gel will be blotted to a membrane filter and hybridized with ^{32}P labelled pPΔCBHII according to the methods in Example 11. Transformants will be identified which have a single copy of the EcoRI fragment from pPΔCBHII integrated precisely at the cbh2 locus. The transformants will also be grown in shaker flasks as in Example 7 and the protein in the culture supernatants examined by isoelectric focusing. In this manner T. reesei GC69 transformants which do not produce the CBHII protein will be generated.

Example 10

Generation of a pyr4$^-$ Derivative of P37PΔCBHI

Spores of the transformant (P37PΔCBHI) which was deleted for the cbh1 gene were spread onto medium containing FOA. A pyr4$^-$ derivative of this transformant was subsequently obtained using the

methods of Example 1. This pyr4- strain was designated P37PΔCBHIPyr-26.

Example 11

Deletion of the cbh2 gene in a strain previously deleted for cbh1

Protoplasts of strain P37PΔCBHIPyr-26 were generated and transformed with EcoRI digested pPΔCBHII according to the methods outlined in Examples 3 and 4.

Purified stable transformants were cultured in shaker flasks as in Example 7 and the protein in the culture supernatants was examined by isoelectric focusing. One transformant (designated P37PΔΔCBH67) was identified which did not produce any CBHII protein. Lane D of FIG. 5 shows the supernatant from a transformant deleted for both the cbh1 and cbh2 genes produced according to the methods of the present invention.

DNA was extracted from strain P37PΔΔCBH67, digested with EcoRI and Asp718, and subjected to agarose gel electrophoresis. The DNA from this gel was blotted to a membrane filter and hybridized with ^{32}p labelled pPΔCBHII (FIG. 7). Lane A of FIG. 7 shows the hybridization pattern observed for DNA from an untransformed T. reesei strain. The 4.1 kb EcoRI fragment containing the wild-type cbh2 gene was observed. Lane B shows the hybridization pattern observed for strain P37PΔΔCBH67. The single 4.1 kb band has been eliminated

and replaced by two bands of approximately 0.9 and 3.1 kb. This is the expected pattern if a single copy of the EcoRI fragment from pPΔCBHII had integrated precisely at the cbh2 locus.

The same DNA samples were also digested with EcoRI and Southern blot analysis was performed as above. In this Example, the probe was ^{32}P labelled pIntCBHII. This plasmid contains a portion of the cbh2 gene coding sequence from within that segment of the cbh2 gene which was deleted in plasmid pPΔCBHII. No hybridization was seen with DNA from strain P37PΔΔCBH67 showing that the cbh2 gene was deleted and that no sequences derived from the pUC plasmid were present in this strain.

Example 12

Construction of pEGIpyr4

The T. reesei egl1 gene, which encodes EGI, has been cloned as a 4.2 kb HindIII fragment of genomic DNA from strain RL-P37 by hybridization with oligonucleotides synthesized according to the published sequence (Penttila et al., 1986, Gene 45:253-263; van Arsdell et al., 1987, Bio/Technology 5:60-64). A 3.6 kb HindIII-BamHI fragment was taken from this clone and ligated with a 1.6 kb HindIII-BamHI fragment containing the T. reesei pyr4 gene obtained from pTpyr2 (see Example 2) and pUC218 (identical to pUC219, see Example 16, but with the multiple cloning site in the opposite orientation) cut with HindIII to give the plasmid pEGIpyr4 (FIG. 8). Digestion of pEGIpyr4 with HindIII would liberate a fragment of DNA containing only T. reesei genomic DNA (the egl1 and pyr4 genes)

except for 24 bp of sequenced, synthetic DNA between the two genes and 6 bp of sequenced, synthetic DNA at one end (see FIG. 8).

Example 13

Transformants of Trichoderma reesei Containing the plasmid pEGIpyr4

A pyr4 defective derivative of T. reesei strain RutC30 (Sheir-Neiss and Montenecourt, (1984), Appl. Microbiol. Biotechnol. 20:46-53) was obtained by the method outlined in Example 1. Protoplasts of this strain were transformed with undigested pEGIpyr4 and stable transformants were purified.

Five of these transformants (designated EP2, EP4, EP5, EP6, EP11), as well as untransformed RutC30 were inoculated into 50 ml of YEG medium (yeast extract, 5 g/l; glucose, 20 g/l) in 250 ml shake flasks and cultured with shaking for two days at 28°C. The resulting mycelium was washed with sterile water and added to 50 ml of TSF medium (0.05M citrate-phosphate buffer, pH 5.0; Avicel microcrystalline cellulose, 10 g/l; KH_2PO_4, 2.0 g/l; $(NH_4)_2SO_4$, 1.4 g/l; proteose peptone, 1.0 g/l; Urea, 0.3 g/l; $MgSO_4.7H_2O$, 0.3 g/l; $CaCl_2$, 0.3 g/l; $FeSO_4.7H_2O$, 5.0 mg/l; $MnSO_4.H_2O$, 1.6 mg/l; $ZnSO_4$, 1.4 mg/l; $CoCl_2$, 2.0 mg/l; 0.1% Tween 80). These cultures were incubated with shaking for a further four days at 28°C. Samples of the supernatant were taken from these cultures and assays designed to measure the total amount of protein and of endoglucanase activity were performed as described below.

The endoglucanase assay relied on the release of soluble, dyed oligosaccharides from Remazol Brilliant Blue-carboxymethylcellulose (RBB-CMC, obtained from MegaZyme, North Rocks, NSW, Australia). The substrate was prepared by adding 2 g of dry RBB-CMC to 80 ml of just boiled deionized water with vigorous stirring. When cooled to room temperature, 5 ml of 2 M sodium acetate buffer (pH 4.8) was added and the pH adjusted to 4.5. The volume was finally adjusted to 100 ml with deionized water and sodium azide added to a final concentration of 0.02%. Aliquots of T. reesei control culture, pEGIpyr4 transformant culture supernatant or 0.1 M sodium acetate as a blank (10-20 µl) were placed in tubes, 250 µl of substrate was added and the tubes were incubated for 30 minutes at 37°C. The tubes were placed on ice for 10 minutes and 1 ml of cold precipitant (3.3% sodium acetate, 0.4% zinc acetate, pH 5 with HCl, 76% ethanol) was then added. The tubes were vortexed and allowed to sit for five minutes before centrifuging for three minutes at approximately 13,000 x g. The optical density was measured spectrophotometrically at a wavelength of 590-600 nm.

The protein assay used was the BCA (bicinchoninic acid) assay using reagents obtained from Pierce, Rockford, Illinois, USA. The standard was bovine serum albumin (BSA). BCA reagent was made by mixing 1 part of reagent B with 50 parts of reagent A. One ml of the BCA reagent was mixed with 50 µl of appropriately diluted BSA or test culture supernatant. Incubation was for 30 minutes at 37°C and the optical density was finally measured spectrophotometrically at a wavelength of 562 nm.

The results of the assays described above are shown in Table 1. It is clear that some of the transformants produced increased amounts of endoglucanase activity compared to untransformed strain RutC30. It is thought that the endoglucanases and exo-cellobiohydrolases produced by untransformed T. reesei constitute approximately 20 and 70 percent respectively of the total amount of protein secreted. Therefore a transformant such as EP5, which produces approximately four-fold more endoglucanase than strain RutC30, would be expected to secrete approximately equal amounts of endoglucanase-type and exo-cellobiohydrolase-type proteins.

The transformants described in this Example were obtained using intact pEGIpyr4 and will contain DNA sequences integrated in the genome which were derived from the pUC plasmid. Prior to transformation it would be possible to digest pEGIpyr4 with HindIII and isolate the larger DNA fragment containing only T. reesei DNA. Transformation of T. reesei with this isolated fragment of DNA would allow isolation of transformants which overproduced EGI and contained no heterologous DNA sequences except for the two short pieces of synthetic DNA shown in FIG. 8. It would also be possible to use pEGIpyr4 to transform a strain which was deleted for either the cbh1 gene, or the cbh2 gene, or for both genes. In this way a strain could be constructed which would over-produce EGI and produce either a limited range of, or no, exo-cellobiohydrolases.

The methods of Example 13 could be used to produce T. reesei strains which would over-produce any of the other cellulase

components, xylanase components or other proteins normally produced by T. reesei.

TABLE 1

Secreted Endoglucanase Activity of T. reesei Transformants

STRAIN	A ENDOGLUCANASE ACTIVITY (O.D. AT 590 nm)	B PROTEIN (mg/ml)	A/B
RutC30	0.32	4.1	0.078
EP2	0.70	3.7	0.189
EP4	0.76	3.65	0.208
EP5	1.24	4.1	0.302
EP6	0.52	2.93	0.177
EP11	0.99	4.11	0.241

The above results are presented for the purpose of demonstrating the overproduction of the EGI component relative to total protein and not for the purpose of demonstrating the extent of overproduction. In this regard, the extent of overproduction is expected to vary with each experiment.

Example 14

Construction of pCEPC1

A plasmid, pCEPC1, was constructed in which the coding sequence for EGI was functionally fused to the promoter from the cbh1

gene. This was achieved using in vitro, site-specific mutagenesis to alter the DNA sequence of the cbh1 and egl1 genes in order to create convenient restriction endonuclease cleavage sites just 5' (upstream) of their respective translation initiation sites. DNA sequence analysis was performed to verify the expected sequence at the junction between the two DNA segments. The specific alterations made are shown in FIG. 9.

The DNA fragments which were combined to form pCEPC1 were inserted between the EcoRI sites of pUC4K and were as follows (see FIG. 10):

A) A 2.1 kb fragment from the 5' flanking region of the cbh1 locus. This includes the promoter region and extends to the engineered BclI site and so contains no cbh1 coding sequence.

B) A 1.9 kb fragment of genomic DNA from the egl1 locus starting at the 5' end with the engineered BamHI site and extending through the coding region and including approximately 0.5 kb beyond the translation stop codon. At the 3' end of the fragment is 18 bp derived from the pUC218 multiple cloning site and a 15 bp synthetic oligonucleotide used to link this fragment with the fragment below.

C) A fragment of DNA from the 3' flanking region of the cbh1 locus, extending from a position approximately 1 kb downstream to approximately 2.5 kb downstream of the cbh1 translation stop codon.

D) Inserted into an NheI site in fragment (C) was a 3.1 kb NheI-SphI fragment of DNA containing the T. reesei pyr4 gene obtained from pTpyr2 (Example 2) and having 24 bp of DNA at one end derived from the pUC18 multiple cloning site.

The plasmid, pCEPC1 was designed so that the EGI coding sequence would be integrated at the cbh1 locus, replacing the coding sequence for CBHI without introducing any foreign DNA into the host strain. Digestion of this plasmid with EcoRI liberates a fragment which includes the cbh1 promoter region, the egl1 coding sequence and transcription termination region, the T. reesei pyr4 gene and a segment of DNA from the 3' (downstream) flanking region of the cbh1 locus (see Fig. 10).

Example 15

Transformants containing pCEPC1 DNA

A pyr4 defective strain of T. reesei RutC30 (Sheir-Neiss, supra) was obtained by the method outlined in Example 1. This strain was transformed with pCEPC1 which had been digested with EcoRI. Stable transformants were selected and subsequently cultured in shaker flasks for cellulase production as described in Example 13. In order to visualize the cellulase proteins, isoelectric focusing gel electrophoresis was performed on samples from these cultures using the method described in Example 7. Of a total of 23 transformants analysed in this manner 12 were found to produce no CBHI protein, which is the expected result of integration of the CEPC1 DNA at the cbh1 locus. Southern blot analysis was used to confirm that integration had indeed occurred at the cbh1 locus in some of these transformants and that no sequences derived from the bacterial plasmid vector (pUC4K) were present (see Fig. 11). For this analysis the DNA from the transformants was digested with PstI before being subjected to

electrophoresis and blotting to a membrane filter. The resulting Southern blot was probed with radiolabelled plasmid pUC4K::cbh1 (see Example 2). The probe hybridised to the cbh1 gene on a 6.5 kb fragment of DNA from the untransformed control culture (FIG. 11, lane A). Integration of the CEPC1 fragment of DNA at the cbh1 locus would be expected to result in the loss of this 6.5 kb band and the appearance of three other bands corresponding to approximately 1.0 kb, 2.0 kb and 3.5 kb DNA fragments. This is exactly the pattern observed for the transformant shown in FIG. 11, lane C. Also shown in FIG. 11, lane B is an example of a transformant in which multiple copies of pCEPC1 have integrated at sites in the genome other than the cbh1 locus.

Endoglucanase activity assays were performed on samples of culture supernatant from the untransformed culture and the transformants exactly as described in Example 13 except that the samples were diluted 50 fold prior to the assay so that the protein concentration in the samples was between approximately 0.03 and 0.07 mg/ml. The results of assays performed with the untransformed control culture and four different transformants (designated CEPC1-101, CEPC1-103, CEPC1-105 and CEPC1-112) are shown in Table 2. Transformants CEPC1-103 and CEPC1-112 are examples in which integration of the CEPC1 fragment had led to loss of CBHI production.

Table 2

Secreted endoglucanase activity of T. reesei transformants

STRAIN	A ENDOGLUCANASE ACTIVITY (O.D. at 590 nm)	B PROTEIN (mg/ml)	A/B
RutC300	0.037	2.38	0.016
CEPC1-101	0.082	2.72	0.030
CEPC1-103	0.099	1.93	0.051
CEPC1-105	0.033	2.07	0.016
CEPC1-112	0.093	1.72	0.054

The above results are presented for the purpose of demonstrating the overproduction of the EGI component relative to total protein and not for the purpose of demonstrating the extent of overproduction. In this regard, the extent of overproduction is expected to vary with each experiment.

It would be possible to construct plasmids similar to pCEPC1 but with any other T. reesei gene replacing the egl1 gene. In this way, overexpression of other genes and simultaneous deletion of the cbh1 gene could be achieved.

It would also be possible to transform pyr4 derivative strains of T. reesei which had previously been deleted for other genes, eg. for cbh2, with pCEPC1 to construct transformants which would, for example, produce no exo-cellobiohydrolases and overexpress endoglucanases.

Using constructions similar to pCEPC1, but in which DNA from another locus of T. reesei was substituted for the DNA from the cbh1 locus, it would be possible to insert genes under the control of another promoter at another locus in the T. reesei genome.

Example 16

Construction of pEGII::P-1

The egl3 gene, encoding EGII (previously referred to as EGIII by others), has been cloned from T. reesei and the DNA sequence published (Saloheimo et al., 1988, Gene 63:11-21). We have obtained the gene from strain RL-P37 as an approximately 4 kb PstI- XhoI fragment of genomic DNA inserted between the PstI and XhoI sites of pUC219. The latter vector, pUC219, is derived from pUC119 (described in Wilson et al., 1989, Gene 77:69-78) by expanding the multiple cloning site to include restriction sites for BglII, ClaI and XhoI. Using methods known in the art the T. reesei pyr4 gene, present on a 2.7 kb SalI fragment of genomic DNA, was inserted into a SalI site within the EGII coding sequence to create plasmid pEGII::P-1 (FIG. 12). This resulted in disruption of the EGII coding sequence but without deletion of any sequences. The plasmid, pEGII::P-1 can be digested with HindIII and BamHI to yield a linear fragment of DNA derived exclusively from T. reesei except for 5 bp on one end and 16 bp on the other end, both of which are derived from the multiple cloning site of pUC219.

Example 17

Transformation of T. reesei GC69 with pEGII::P-1 to creat a strain unable to produce EGII

T. reesei strain GC69 will be transformed with pEGII::P-1 which had been previously digested with HindIII and BamHI and stable transformants will be selected. Total DNA will be isolated from the transformants and Southern blot analysis used to identify those transformants in which the fragment of DNA containing the pyr4 and egl3 genes had integrated at the egl3 locus and consequently disrupted the EGII coding sequence. The transformants will be unable to produce EGII. It would also be possible to use pEGII::P-1 to transform a strain which was deleted for either or all of the cbh1, cbh2, or egl1 genes. In this way a strain could be constructed which would only produce certain cellulase components and no EGII component.

Example 18

Transformation of T. reesei with pEGII::P-1 to create a strain unable to produce CBHI, CBHII and EGII

A pyr4 deficient derivative of strain P37PΔΔCBH67 (from Example 11) was obtained by the method outlined in Example 1. This strain P37PΔΔ67P-1 was transformed with pEGII::P-1 which had been previously digested with HindIII and BamHI and stable transformants were selected. Total DNA was isolated from transformants and Southern blot analysis used to identify strains in which the fragment of DNA containing the pyr4 and egl3 genes had integrated at the egl3 locus and consequently disrupted the EGII coding sequence. The

Southern blot illustrated in FIG. 13 was probed with an approximately 4 kb PstI fragment of T. reesei DNA containing the egl3 gene which had been cloned into the PstI site of pUC18 and subsequently re-isolated. When the DNA isolated from strain P37PΔΔ67P 1 was digested with PstI for Southern blot analysis the egl3 locus was subsequently visualized as a single 4 kb band on the autoradiograph (FIG. 13, lane E). However, for a transformant disrupted for the egl3 gene this band was lost and was replaced by two new bands as expected (FIG. 13, Lane F). If the DNA was digested with EcoRV or BglII the size of the band corresponding to the egl3 gene increased in size by approximately 2.7 kb (the size of the inserted pyr4 fragment) between the untransformed P37PΔΔ67P 1 strain (Lanes A and C) and the transformant disrupted for egl3 (FIG. 13, Lanes B and D). The transformant containing the disrupted egl3 gene illustrated in FIG. 13 (Lanes B, D and F) was named A22. The transformant identified in FIG. 13 is unable to produce CBHI, CBHII or EGII.

Example 19

Construction of pPΔEGI-1

The egl1 gene of T. reesei strain RL-P37 was obtained, as described in Example 12, as a 4.2 kb HindIII fragment of genomic DNA. This fragment was inserted at the HindIII site of pUC100 (a derivative of pUC18; Yanisch-Perron et al., 1985, Gene 33:103-119, with an oligonucleotide inserted into the multiple cloning site adding restriction sites for BglII, ClaI and XhoI). Using methodology known in the art an approximately 1 kb EcoRV fragment extending from a

position close to the middle of the EGI coding sequence to a position beyond the 3' end of the coding sequence was removed and replaced by a 3.5 kb ScaI fragment of T. reesei DNA containing the pyr4 gene. The resulting plasmid was called pPΔEGI-1 (see Fig. 14).

The plasmid pPΔEGI-1 can be digested with HindIII to release a DNA fragment comprising only T. reesei genomic DNA having a segment of the egl1 gene at either end and the pyr4 gene replacing part of the EGI coding sequence, in the center.

Transformation of a suitable T. reesei pyr4 deficient strain with the pPΔEGI-1 digested with HindIII will lead to integration of this DNA fragment at the egl1 locus in some proportion of the transformants. In this manner a strain unable to produce EGI will be obtained.

Example 20

Construction of pΔEGIpyr-3 and Transformation of a pyr4 deficient strain of T. reesei

The expectation that the EGI gene could be inactivated using the method outlined in Example 19 is strengthened by this experiment. In this case a plasmid, pΔEGIpyr-3, was constructed which was similar to pPΔEGI-1 except that the Aspergillus niger pyr4 gene replaced the T. reesei pyr4 gene as selectable marker. In this case the egl1 gene was again present as a 4.2 kb HindIII fragment inserted at the HindIII site of pUC100. The same internal 1 kb EcoRV fragment was removed as during the construction of pPΔEGI-1 (see Example 19) but in this

case it was replaced by a 2.2 kb fragment containing the cloned A. niger pyrG gene (Wilson et al., 1988, Nucl. Acids Res. 16 p.2339). Transformation of a pyr4 deficient strain of T. reesei (strain GC69) with pΔEGIpyr-3, after it had been digested with HindIII to release the fragment containing the pyrG gene with flanking regions from the egl1 locus at either end, led to transformants in which the egl1 gene was disrupted. These transformants were recognized by Southern blot analysis of transformant DNA digested with HindIII and probed with radiolabelled pΔEGIpyr-3. In the untransformed strain of T. reesei the egl1 gene was present on a 4.2 kb HindIII fragment of DNA and this pattern of hybridization is represented by Fig. 15, lane C. However, following deletion of the egl1 gene by integration of the desired fragment from pΔEGIpyr-3 this 4.2 kb fragment disappeared and was replaced by a fragment approximately 1.2 kb larger in size, FIG. 15, lane A. Also shown in FIG. 15, lane B is an example of a transformant in which integration of a single copy of pPΔEGIpyr-3 has occurred at a site in the genome other than the egl1 locus.

Example 21

Transformation of T.reesei with pPΔEGI-1 to create a strain unable to produce CBHI, CBHII, EGI and EGII

A pyr4 deficient derivative of strain A22 (from Example 18) will be obtained by the method outlined in Example 1. This strain will be transformed with pPΔEGI-1 which had been previously digested with HindIII to release a DNA fragment comprising only T. reesei genomic

DNA having a segment of the egl1 gene at either end with part of the EGI coding sequence replaced by the pyr4 gene.

Stable pyr4+ transformants will be selected and total DNA isolated from the transformants. The DNA will be probed with ^{32}P labelled pPΔEGI-1 after Southern blot analysis in order to identify transformants in which the fragment of DNA containing the pyr4 gene and egl1 sequences has integrated at the egl1 locus and consequently disrupted the EGI coding sequence. The transformants identified will be unable to produce CBHI, CBHII, EGI and EGII.

Example 22

Cloning and identification of the Low pI and High pI Xylanases genes of T. reesei

Two different xylanase enzymes from T. reesei were purified starting with CYTOLASE 123™ (a complete fungal cellulase enzyme composition obtained from T. reesei and available from Genencor International, Inc., South San Francisco, CA). The substrate used in assays for xylanase activity was 4-O-Methyl-D-glucurono-D-xylan Remazol Brilliant Blue R (MegaZyme, North Rocks, N.S.W., Australia). Fractionations were done using columns containing the following resins: Sephadex G-25 gel filtration resin (Sigma Chemical Company, St. Louis, MO), QA Trisacryl M anion exchange resin and SP Trisacryl M cation exchange resin (IBF Biotechnics, Savage, MD). CYTOLASE 123™, (0.5 grams) was desalted using a column of 3 liters of Sephadex G-25 gel filtration resin equilibrated with 10mM sodium

phosphate buffer at pH 6.8. The desalted solution was then loaded onto a column of 20 ml of QA Trisacryl M anion exchange resin. The fraction bound on this column contained the low pI xylanase (pI = 5.2). The low pI xylanase protein was eluted by gradient elution using an aqueous gradient containing from 0 to 500 mM sodium chloride. The fraction not bound on this column contained the high pI xylanase (pI = 9.0). This fraction was desalted using a column of Sephadex G-25 gel filtration resin equilibrated with 10 mM sodium citrate, pH 3.3. This solution was then loaded onto a column of 20 ml of SP Trisacryl M cation exchange resin. The high pI xylanase was eluted using an aqueous gradient containing from 0 to 200 mM sodium chloride.

Each xylanase protein was precipitated by the addition of 0.9 ml of acetone to 0.1 ml of enzyme solution (at a concentration of 1 mg/ml) and incubation at -20°C for 10 minutes. The protein was collected by centrifugation and the pellet dried and resuspended in 0.05 ml of 100 mM Tris with the pH adjusted to 8.0 with TFA (trifluoroacetic acid) and 2M urea. Five μg of trypsin/chymotrypsin was added and the mixture incubated at 37°C for four hours.

Individual peptides were purified on a HPLC (high pressure liquid chromatography) column. A Synchropak RP-4 column was equilibrated in milliQ water with 0.05% TEA (triethylamine) and 0.05% TFA. The sample was loaded onto the HPLC column and elution was carried out with 100% acetonitrile and 0.05% TEA and 0.05% TFA, with a gradient of 1% per minute. The amino-terminal regions of isolated peptides were sequenced by the method of Edman using a fully automated apparatus.

1) Low pI Xylanase gene

A degenerate pool of oligonucleotides was made corresponding to a region (Tyr Ile Met Glu Asp Asn His Asn Tyr) within one of the sequenced peptides. Southern blots of T. reesei genomic DNA digested with HindIII and other restriction enzymes were probed with the ^{32}P labelled oligonucleotide pool. A 2 kb HindIII fragment was observed to hybridize with the oligonucleotide pool. The 2 kb HindIII fragment was isolated from a plasmid bank of T. reesei HindIII fragments contained in pUC219 using the radioactively labelled oligonucleotide pool as a probe. DNA sequencing near one end of the 2 kb HindIII fragment revealed a translated protein sequence that was identical to the entire sequence obtained from one of the peptides (peptide 1) from the low pI xylanase protein. Another translated protein sequence close to the previous sequence was found to be highly similar to the protein sequence from two different xylanase enzymes from a Bacillus species. The radioactively labelled 2 kb HindIII fragment was used as a probe in Southern blots of restriction enzyme-digested T. reesei genomic DNA to construct a restriction map of the region around the 2 kb HindIII fragment. Based on this data, a 3 kb SphI - BamHI fragment was then isolated from a library of T. reesei SphI - BamHI fragments contained in pUC219 using the 2 kb HindIII fragment as a probe. DNA sequencing, by methods known in the art, within the 3 kb SphI - BamHI fragment revealed a deduced protein sequence matching that derived from the second sequenced peptide (peptide2) of the low pI xylanase which confirmed that the gene for the low pI xylanase had been cloned. Preliminary DNA sequence data, when converted to a protein sequence, shows extensive regions of similarity of the low pI xylanase to xylanases from two different

Bacillus species obtained from a publicly available data bank, and to a sequence within the partially cloned high pI xylanase gene (see FIG. 16).

2) High pI Xylanase gene

Two degenerate pools of oligonucleotides, one consisting of 128 oligomers 27 bp in length (10 bp corresponding to an EcoRI restriction site followed by 17 bp coding for the amino acid sequence Gly Trp Gln Pro Gly Thr of peptide 1) and the other pool containing 96 oligomers 27 bp in length (10 bp corresponding to a PstI restriction site followed by 17 bp coding for the reverse complement to the sequence Ile Val Glu Asn Phe Gly of peptide 2) were created by methods known in the art and were used as primers in a polymerase chain reaction (PCR) on T. reesei genomic DNA. After polyacrylamide gel electrophoresis, an approximately 260 bp fragment was observed. After digestion with EcoRI and PstI, the fragment was subcloned into M13mp19 for DNA sequencing. The deduced amino acid sequence at the 5' end of this fragment was identical to peptide 1. The deduced amino acid sequence, interrupted by a 108 bp intron, showed a high degree of similarity to the protein sequences of xylanases from Bacillus circulans and Bacillus pumilus (see FIG. 16). When a Southern blot of T. reesei genomic DNA digested with Asp718 was probed with the radioactively labelled 260 bp fragment a single 5 kb band was seen.

The two cloned T. reesei xylanase genes will be fully characterized in order to ascertain the complete nucleotide sequence of the coding region, as well as the sequence of upstream and downstream regions. The position of introns and the 5' and 3' ends of

the transcribed region will be determined by sequence analysis of corresponding cDNA clones using methods known in the art. A map of restriction endonuclease sites within the gene and its flanking regions will be generated. Using the above data it will be possible using methods set forth in Examples 12 and 14 to construct plasmids similar to pCEPC1 or pEGIpyr4 but with either one of the xylanase genes substituted for the egl1 gene in these constructions. Transformation of appropriate T. reesei strains with a substantially homologous DNA fragment containing a xylanase gene and a selectable marker by the methods set forth in Examples 3 and 4 will allow extra copies of either or both xylanase genes to be inserted into the T. reesei genome, either at the cbh1 locus or elsewhere, and thus achieve overexpression of the xylanase genes. In this way T. reesei transformants will be obtained which overexpress either or both the high pI xylanase protein and the low pI xylanase protein. Additionally, T. reesei strains will be created which overexpress the low pI and/or high pI xylanase genes and which are unable to produce any or all of the cellulase components using the methods described in this application.

Using the methods set forth in Example 2 plasmids will be constructed in which all or part of the xylanase coding region will be deleted and replaced with a selectable marker such as the pyr4 gene. Alternatively, the pyr4 gene could be inserted into the xylanase gene disrupting the coding region by the method shown in Example 16. A linear substantially homologous DNA fragment containing the selectable marker flanked by sequences will be used to transform a T. reesei strain. In this way transformants will be created which are unable to produce a functional high pI or low pI xylanase or both.

While the invention has been described in terms of various preferred embodiments, the skilled artisan will appreciate that various modifications, substitutions, omissions, and changes may be made without departing from the scope and spirit thereof. Accordingly, it is intended that the scope of the present invention be limited solely by the scope of the following claims, including equivalents thereof.

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WE CLAIM:

1. A process for transforming T. reesei, said process comprising the steps of:

(a) treating T. reesei cells or protoplasts with substantially homologous recombinant DNA under conditions permitting at least some of said T. reesei cells to take up said substantially homologous recombinant DNA and form transformants therewith; and

(b) obtaining T. reesei transformants.

2. The process according to Claim 1, wherein said substantially homologous recombinant DNA is in a form of linear fragments.

3. The process according to Claim 2, wherein said substantially homologous recombinant DNA contains a predetermined selectable marker gene.

4. The process according to Claim 2, wherein said T. reesei strain lacks the function of a selectable marker gene and said substantially homologous recombinant DNA contains said predetermined selectable marker gene.

5. The process according to Claim 3, wherein said selectable marker is a gene which encodes for an measurable product.

6. The process according to Claim 3, wherein said selectable marker is an orotidine 5' monophosphate decarboxylase gene (pyr4).

7. The process according to Claim 1, wherein said T. reesei cells are T. reesei strain GC69.

8. The process according to Claim 1, wherein said T. reesei transformants lack a part of a gene or genes that encode a protein or proteins.

9. The process according to Claim 1 wherein said T. reesei transformants lack a part of a gene or genes that encode cellulase enzymes.

10. The process according to Claim 1, wherein said T. reesei transformants do not produce one or more functional cellulase components said components being selected from the group comprising CBHI, CBHII, EGI, EGII, EGIII and mixtures thereof.

11. The process according to Claim 2, wherein said substantially homologous recombinant DNA is the linear substantially homologous DNA fragment which encodes a selectable marker flanked by DNA from the T. reesei cbh1 locus.

12. The process according to Claim 11, wherein said T. reesei transformants do not produce a functional CBHI cellulase component.

13. The process according to Claim 2, wherein said substantially homologous recombinant DNA is a substantially homologous DNA fragment which encodes a selectable marker flanked by DNA from the T. reesei cbh2 gene.

14. The process according to Claim 13, wherein said T. reesei transformants do not produce a functional CBHII cellulase component.

15. The process according to Claim 2, wherein said substantially homologous recombinant DNA is a substantially homologous DNA fragment encoding a selectable marker flanked by DNA from the egl3 gene.

16. The process according to Claim 15, wherein said T. reesei transformants do not produce an functional EGII cellulase component.

17. The process according to Claim 2, wherein said substantially homologous recombinant DNA is a substantially homologous DNA fragment encoding a selectable marker flanked by DNA from the egl1 gene.

18. The process according to Claim 17, wherein said T. reesei transformants do not produce a functional EGI cellulase component.

19. The process according to Claim 1, wherein said T. reesei transformants do not produce a functional low pI xylanase protein.

20. The process according to Claim 1, wherein said T. reesei transformants do not produce a functional high pI xylanase protein.

21. The process according to Claim 1, wherein said T. reesei transformants overexpress a protein or proteins.

22. The process according to Claim 1, wherein said T. reesei transformants overexpress an enzyme or enzymes.

23. The process according to Claim 2, wherein said substantially homologous recombinant DNA is a substantially homologous DNA fragment which encodes a selectable marker and the EGI protein.

24. The process according to Claim 23, wherein said T. reesei transformants overexpress an EGI cellulase component.

25. The process according to Claim 2, wherein said substantially homologous recombinant DNA is a substantially homologous DNA fragment encoding a selectable marker and the EGI protein and flanked by DNA from the cbh1 locus.

26. The process according to Claim 25, wherein said T. reesei transformants do not produce a functional CBHI cellulase component and overexpress an EGI cellulase component.

27. The process according to Claim 1, wherein said T. reesei transformants overexpress a xylanase protein.

28. The process according to Claim 2, wherein said substantially homologous recombinant DNA is a substantially homologous DNA fragment which encodes a selectable marker and the high pI xylanase protein.

29. The process according to Claim 28, wherein said T. reesei transformants overexpress the high pI xylanase protein.

30. The process according to Claim 2, wherein said substantially homologous recombinant DNA is a substantially homologous DNA fragment which encodes a selectable marker and the low pI xylanase protein.

31. The process according to Claim 30, wherein said T. reesei transformants overexpress the low pI xylanase protein.

32. A protein composition which composition is substantially free of heterologous protein obtained by the process of:

(a) treating T. reesei cells with substantially homologous recombinant DNA under conditions permitting at least some of said T. reesei cells to take up said DNA;

(b) obtaining T. reesei transformants; and

(c) isolating a protein composition produced from said transformants.

33. The protein composition according to Claim 32, wherein said protein composition is a cellulase composition which does not contain one or more functional cellulase components.

34. The protein composition according to Claim 32 wherein said protein composition is a cellulase composition which does not contain one or more of functional CBHI, CBHII, EGI, EGII and EGIII components and mixtures thereof.

35. The protein composition according to Claim 32, wherein said protein composition is a xylanase composition which does not contain one or more functional xylanase proteins.

36. The protein composition according to Claim 32 wherein said protein composition is a xylanase composition which does not contain one or more of functional CBHI, CBHII, EGI, EGII and EGIII components and mixtures thereof.

37. A cellulase composition derived from T. reesei which does not contain cellulase components selected from the group comprising one or more of functional CBHI, CBHII, EGI, EGII and EGIII components and which composition is substantially free of heterologous proteins.

38. The cellulase composition according to Claim 37, wherein said cellulase composition does not contain a functional CBHI component.

39. The cellulase composition according to Claim 37, wherein said cellulase composition does not contain a functional CBHII component.

40. The cellulase composition according to Claim 37, wherein said cellulase composition does not contain a functional EGI component.

41. The cellulase composition according to Claim 37, wherein said cellulase composition does not contain a functional EGII component.

42. The cellulase composition according to Claim 37, wherein said cellulase composition does not contain a functional EGIII component.

43. A cellulase compostion which composition is substantially free of heterologous protein obtained by the process of:

(a) treating T. reesei cells with substantially homologous linear recombinant DNA fragments from the group comprising:

i) DNA coding for a selectable marker flanked by DNA from the cbh1 locus;

ii) DNA coding for a selectable marker flanked by DNA from the cbh2 locus;

iii) DNA coding for a selectable marker flanked by DNA from the egl1 locus; and

iv) DNA coding for a selectable marker flanked by DNA from the egl3 locus;

under conditions permitting at least some of said T. reesei cells to take up said DNA;

(b) obtaining T. reesei transformants which are unable to produce functional CBHI, CBHII, EGI, EGII components: and

(c) isolating a cellulase composition produced from said transformants which does not contain functional CBHI, CBHII, EGI, EGII components.

44. Transformed T. reesei cells containing substantially homologous DNA and which do not produce a functional cellulase component.

45. Transformed T. reesei cells containing substantially homologous DNA and which do not produce functional cellulase components selected from the group of CBHI, CBHII, EGI, EGII, EGIII and mixtures therof.

46. Transformed T. reesei cells containing substantially homologous DNA and which do not produce a functional CBHI component.

47. Transformed T. reesei cells containing substantially homologous DNA and which do not produce a functional CBHII component.

48. Transformed T. reesei cells containing substantially homologous DNA and which do not produce a functional EGI component.

49. Transformed T. reesei cells containing substantially homologous DNA and which do not produce a functional EGII component.

50. Transformed T. reesei cells containing substantially homologous DNA and which do not produce a functional low pI xylanase protein.

51. Transformed T. reesei cells containing substantially homologous DNA and which do not produce a functional high pI xylanase protein.

52. Transformed T. reesei cells containing substantially homologous DNA and which overexpress a functional EGI cellulase component.

53. Transformed T. reesei cells containing substantially homologous DNA and which overexpress a functional high pI xylanase protein.

54. Transformed T. reesei cells containing substantially homologous DNA and which overexpress a functional low pI xylanase protein.

55. A recombinant DNA construct which contains a selectable marker gene and all or part of the T. reesei cbh1 gene.

56. A plasmid which contains the recombinant DNA construct of claim 55.

57. A recombinant DNA construct which contains a selectable marker gene and all or part of the T. reesei cbh2 gene.

58. A plasmid which contains the recombinant DNA construct of claim 57.

59. A recombinant DNA construct which contains a selectable marker gene and all or part of the T. reesei egl1 gene.

60. A plasmid which contains the recombinant DNA construct of claim 59.

61. A recombinant DNA construct which contains a selectable marker gene and all or part of the T. reesei egl3 gene.

62. A plasmid which contains the recombinant DNA construct of claim 61.

63. A recombinant DNA construct which contains a selectable marker gene and all or part of the T. reesei low pI xylanase gene.

64. A plasmid which contains the recombinant DNA construct of claim 63.

65. A recombinant DNA construct which contains a selectable marker gene and all or part of the T. reesei high pI xylanase gene.

66. A plasmid which contains the recombinant DNA construct of claim 65.

67. A T. reesei gene which codes for the low pI xylanase protein.

68. A T. reesei gene which codes for the high pI xylanase protein.

69. A substantially purified T. reesei low pI xylanase protein.

70. A substantially purified T. reesei low pI xylanase protein further comprising the sequence set forth in FIG. 16.

71. A substantially purified T. reesei high pI xylanase protein.

72. A substantially purified T. reesei high pI xylanase protein further comprising the sequence set forth in FIG. 16.

73. A process for purifying the low pI xylanase protein of T. reesei comprising:

a) loading a cytolase solution onto a column of QA Trisacryl anion exchange resin; and

b) eluting said low pI xylanase.

74. A process for purifying the high pI xylanase protein of T. reesei comprising:

a) loading a cytolase solution onto a column of QA Trisacryl anion exchange resin;

b) collecting a flow-through; and

c) loading the flow-through onto a cation exchange resin and eluting said high pI xylanase.

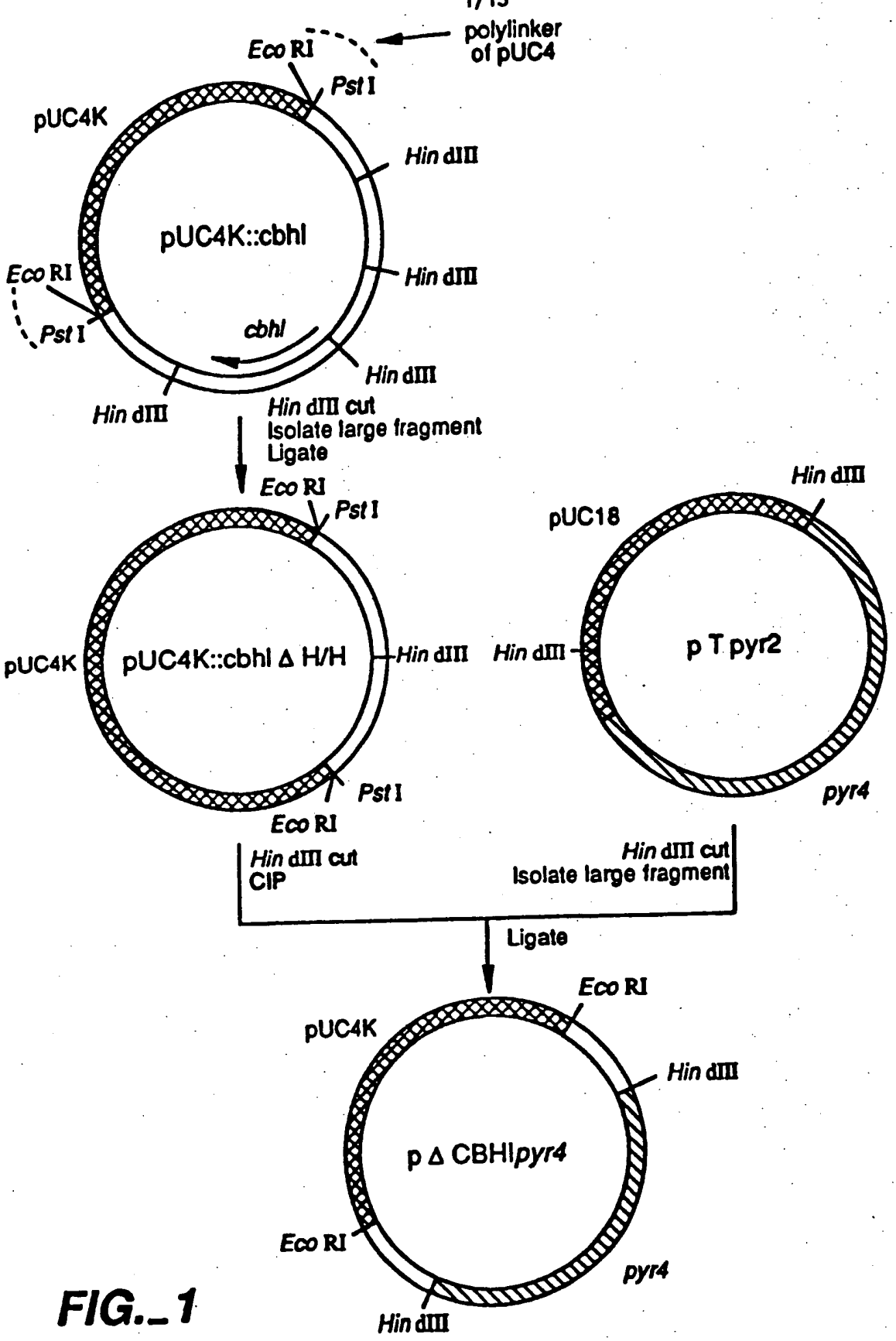
polylinker of pUC4
Eco RI
Pst I
pUC4K
Hin dIII
pUC4K::cbhI
Eco RI
Pst I
cbhI
Hin dIII
Hin dIII
Hin dIII cut
Isolate large fragment
Ligate
Eco RI
Pst I
pUC4K
pUC4K::cbhI Δ H/H
Hin dIII
Pst I
Eco RI
Hin dIII
pUC18
Hin dIII
p T pyr2
pyr4
Hin dIII cut
CIP
Hin dIII cut
Isolate large fragment
Ligate
Eco RI
pUC4K
Hin dIII
p Δ CBHIpyr4
Eco RI
pyr4
Hin dIII

FIG._1

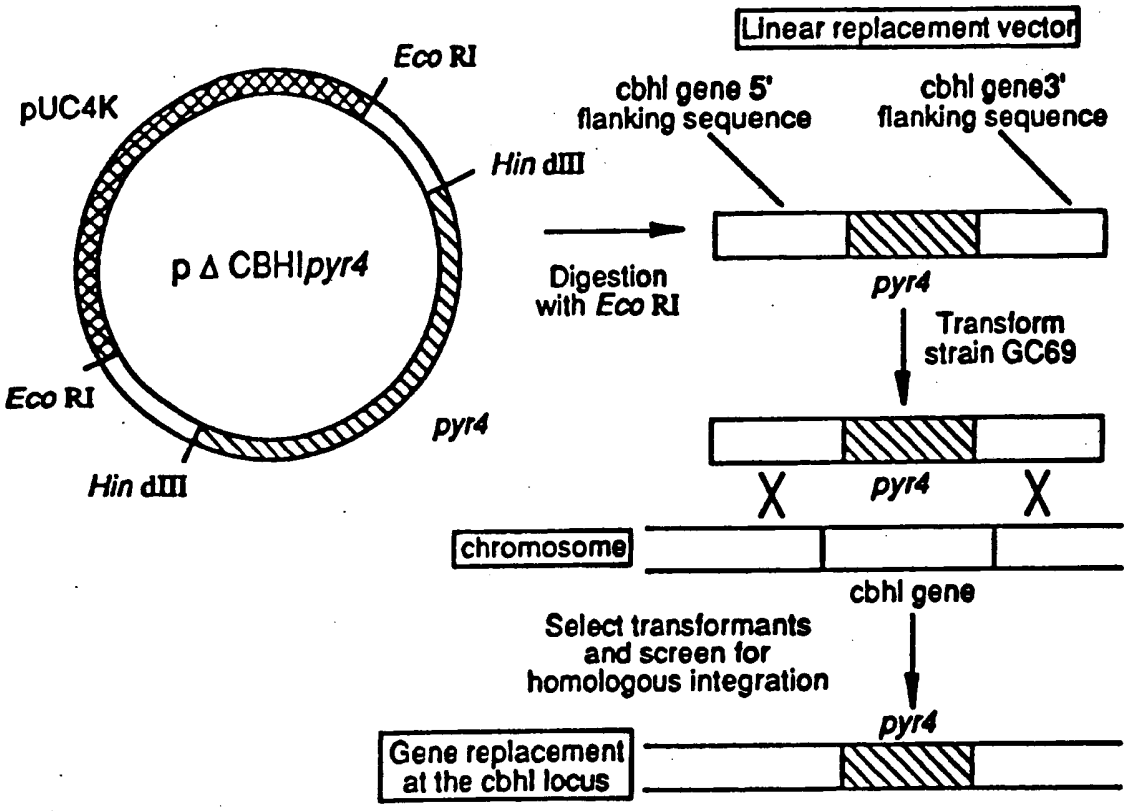
pUC4K
Eco RI
Hin dIII
p Δ CBHIpyr4
Eco RI
Hin dIII
pyr4
Linear replacement vector
cbhl gene 5' flanking sequence
cbhl gene3' flanking sequence
Digestion with Eco RI
pyr4
Transform strain GC69
pyr4
chromosome
cbhl gene
Select transformants and screen for homologous integration
pyr4
Gene replacement at the cbhl locus

FIG._2

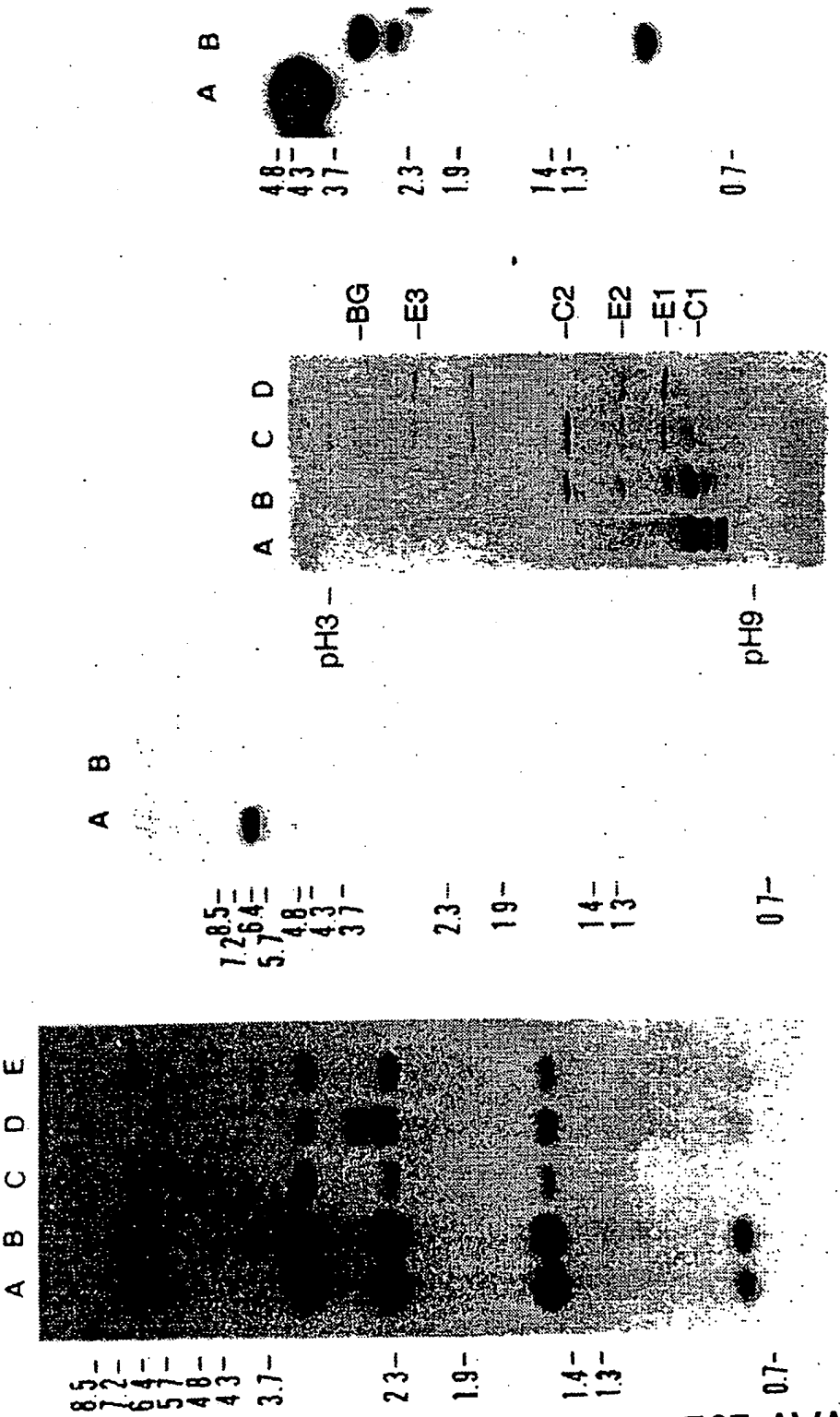

FIG_3

FIG_4

FIG_5

FIG_7

BEST AVAILABLE COPY


Eco RI
Cla I
Eco RI
1.7 kb
Hin dIII
CBHII CODING REGION
0.7 kb
Eco RI

FIG._6A

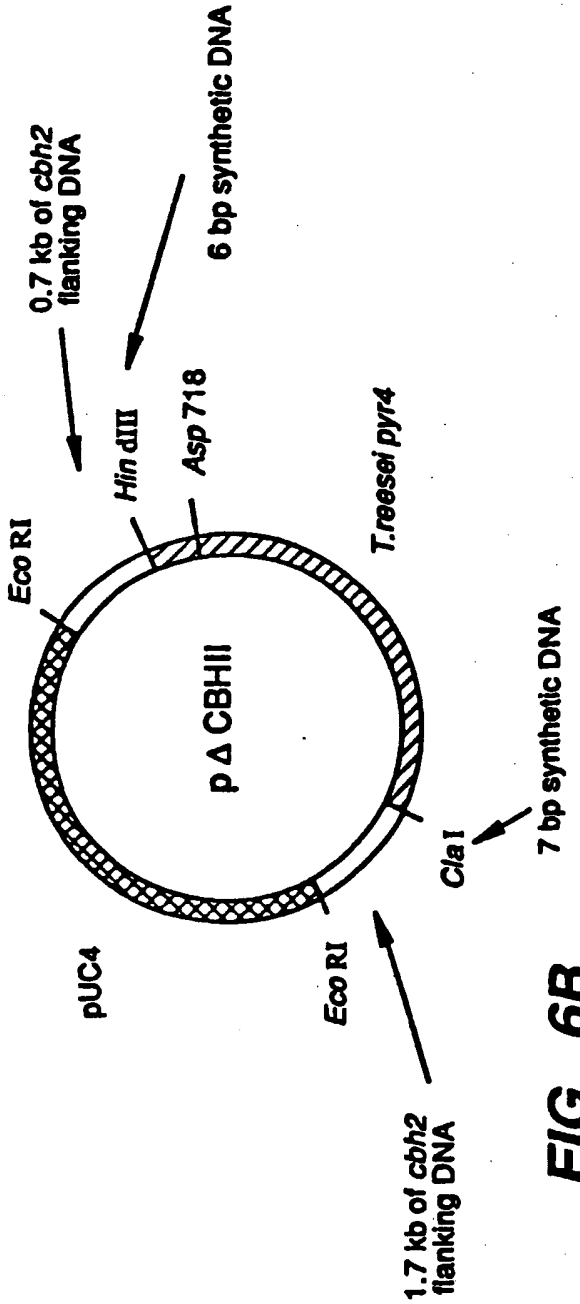
0.7 kb of cbh2 flanking DNA
6 bp synthetic DNA
Hin dIII
Asp 718
T.reesei pyr4
Eco RI
p Δ CBHII
Cla I
7 bp synthetic DNA
pUC4
Eco RI
1.7 kb of cbh2 flanking DNA

FIG._6B

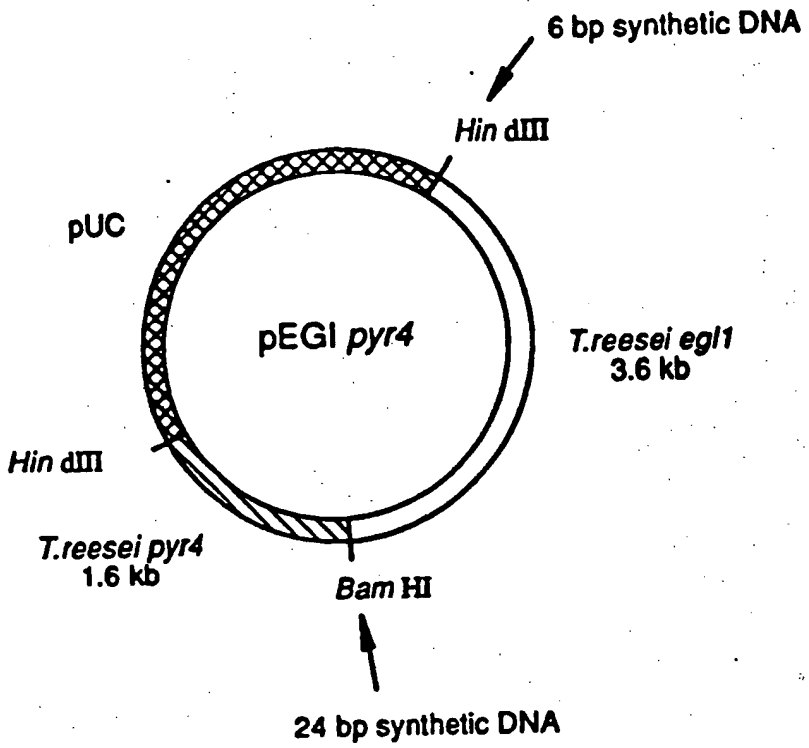
6 bp synthetic DNA
Hin dIII
pUC
pEGI pyr4
T.reesei egl1
3.6 kb
Hin dIII
T.reesei pyr4
1.6 kb
Bam HI
24 bp synthetic DNA

FIG._8

cbh1

```
AAACCCAATAGTCAACCGCGGACTGGCAT ATG TAT CGG
                G T A
AAACCCAATAGTGATCAGCGGACTGGCAT ATG TAT CGG
          BclI                First 3 codons
```

egl1

```
TAGTCCTTCTTGTTGTCCCAAA ATG GCG CCC
              GGA
TAGTCCTTCTTGGGATCCCAAA ATG GCG CCC
          BamHI      First 3 codons
```

FIG. 9

LINEAR FRAGMENT OF DNA OBTAINED FROM pCEPC1 BY EcoRI DIGESTION

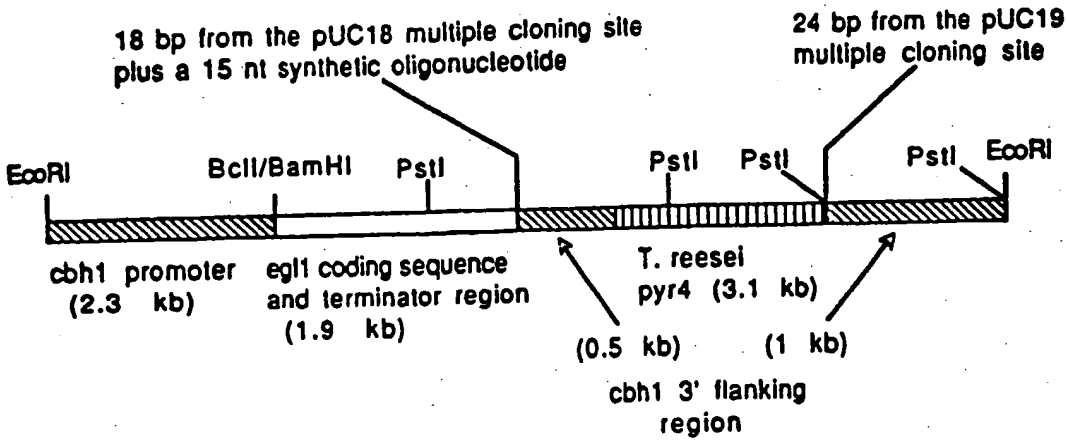

FIG._10

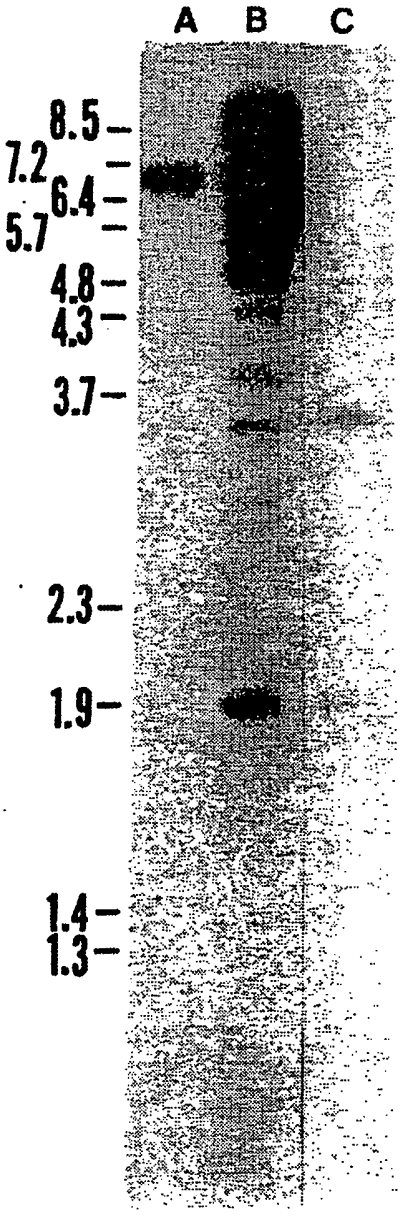

FIG_11

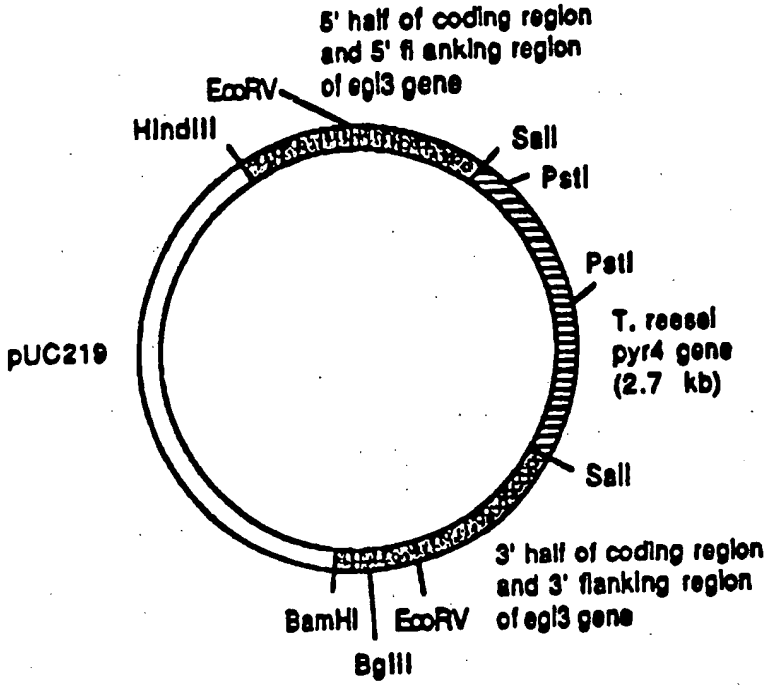

DIAGRAM OF pEGII::P-1

FIG._12

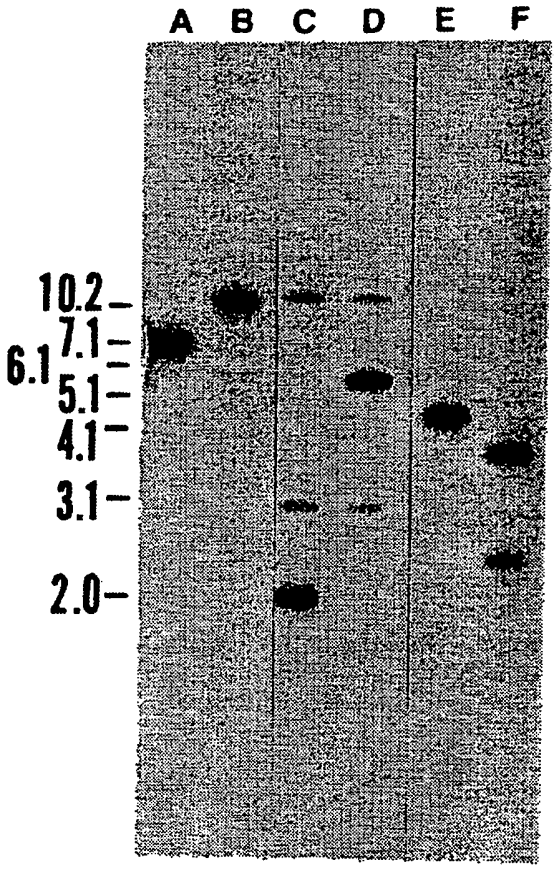

FIG_13

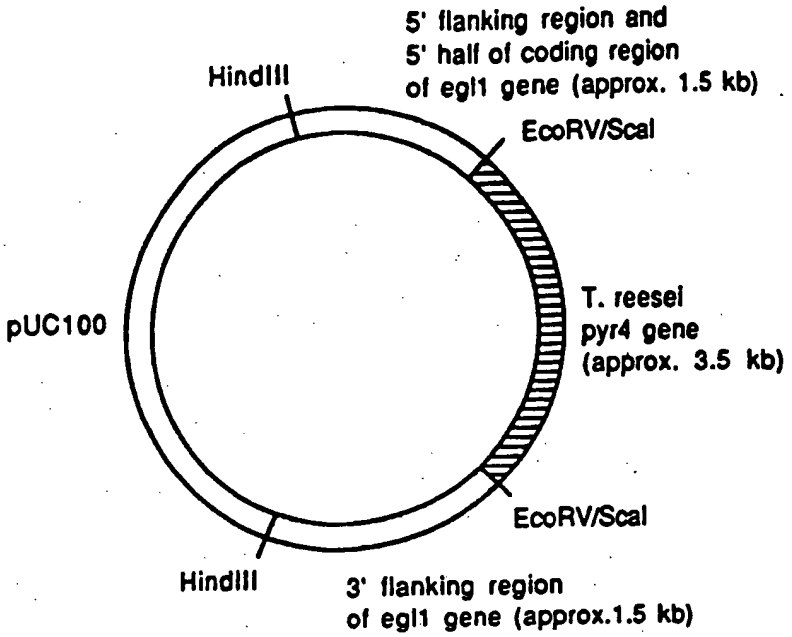
5' flanking region and
5' half of coding region
of egl1 gene (approx. 1.5 kb)
HindIII
EcoRV/ScaI
pUC100
T. reesei
pyr4 gene
(approx. 3.5 kb)
EcoRV/ScaI
HindIII
3' flanking region
of egl1 gene (approx.1.5 kb)

FIG._14

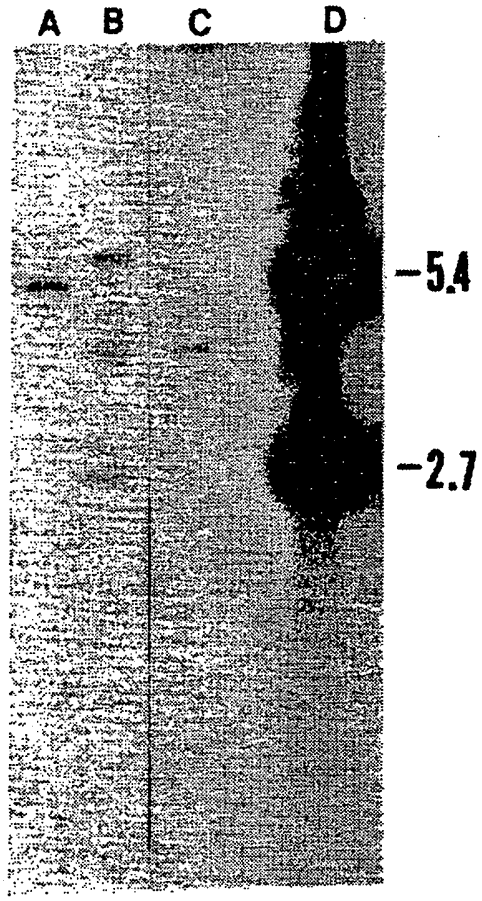

FIG_15

```
 * :=>  match across all seqs.
 . :=>  conservative substitutions

low pI                                                          MVAFSSLICALTT
trichv                                                          -------------
baccir                                                          -------------
bacpum                                                          -------------

low pI          IASTLAMPTGLEPESSVNVTERGMYDFVGAHNDHRRRASINY-DQNYQTGGQVSYSPSNT
trichv                                    QSIGP-GTGFNNGYFYSYWNDGHGGVTYTNGPG
baccir          MF--KFKKNFL--VGLSAALMSISLFSATASA--ASTD--Y-WQNWTDGGGIVNAVNGSG
bacpum          MNLRKLRLLFVMCIGLTLILTAVPAHARTITNNEMGNHSGYDYELWKDYGNTSMTLN-NG

highpI                               ~GWQPGTKNK-----VINFSGSY-NPNGNSYLSVYGWSRNPL
low pI          G-FSVNWNTQDD--FVVGVGWTTGSSA------PINFGGSFSVNSGTGLLSVYGWSTNPL
trichv          GQFSVNWSNSGN--FVGGKGWQPGTKNK-----VINFSGSY-NPNGNSYLSVYGWSRNPL
baccir          GNYSVNWSNTGN--FVVGKGWTTGSPFR-----TINYNAGVWAPNGNGYLTLYGWTRSPL
bacpum          GAFSAGWNNIGNALFRKGKKFDSTRTHHQLGNISINYNAS-FNPSGNSYLCVYGWTQSPL
                * .*..*       *  *                 **        .* . * .***   **

highpI          IEYYIVENFG~
low pI          VEYYIMEDNHNY-PAQGTVK-GTVTSDGATYTIWENTRVNEPSIQG-TATFNQYISVRNSPR
trichv          IEYYIVENFGTYNPSTGATKLGEVTSDGSVYDIYRTQRVNQPSIIG-TAT-YQYWSVRRTHR
baccir          IEYYVVDSWGTYRP-TGTYK-GTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQSKR
bacpum          AEYYIVDSWGTYRP-TGAYK-GSFYADGGTYDIYETTRVNQPSIIGI-ATFKQYWSVRQTKR
                 ***.      * *  *  *.* .  **  * *    * * *** *   *  ** ***   *

low pI          TSG---TVTVQNHFNAWASLGCTLGR~
trichv          SSG---SVNTAN-FNAWAQQGLTLGTMD-YVQIVAVEGYFSSGSASITV
baccir          PTGSNATITFTNHVNAWKSHGMNLGSNWAYQV-MATEGYQSSGSSNVTVW------
bacpum          TSG---TVSVSAHFRKWESLGMPMGK--MYETAFTVEGYQSSGSANVMTNQLFIGN
                ..*    .. ..  . *.  *  .*.   *.. ...********.** .
```

FIG._16

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/07269

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12P 21/00; C12N 1/14, 9/58, 15/80

US 435/69.1, 183,223,254,320.1; 530/371,412

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S. Cl.	435/69.1,183,223,254,320.1; 530/371,412

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS, BIOSIS, CAS,

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Journal of Electron Microscopic Techniques, Vol. 8, No. 4, April 1988, R. H. Berg. et al. "How do Trichoderma reesi cellobiohyrolases bind to and degrade cellulose", pages 371-379, see abstract only.	1-74
A	Trichoderma reesi Cellulases, issued 1990, T. T. Teeri et al. "Engineering Trichoderma and its cellulases-Trichoderma reesi cellulase and cellobiochydrolase gene cloning and expression: potential strain and improvement and enzyme engineering", pages 156-167, see abstract only.	1-74
Y	Recent Advances in Biotechnology and Applied Biology, issued 1988, J. K. C. Knowles, "The use of gene technology in the development of novel cellulolytic organisms- Trichoderma reesi cellulase and cellulobihydrolase gene cloning and expression; a review., pages 139-142, see abstract only.	1-74

* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
28 January 1992	02 MAR 1992
International Searching Authority	**Signature of Authorized Officer**
ISA/US	John Leguyader tf

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A,P	FEBS Letters, Vol. 291, No. 1, issued October 1991, S. Aho, "Structureal and functional analysis of Trichoderma reesi endoglucanase I expressed in yeast Saccharomyces cerevisiae", page 45-49.	1-74
A,P	Journal of Biotechnology, Vol. 20., issued 1991, E.M. Kubicek-Pranz et al., "Transformation of Trichoderma reesi with the cellobiohydrolase II gene as a means for obtaining strains with increased cellulase production and specific activity", pages 83-93.	1-74
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Y	Gene, Vol. 45, issued 1986, Penttila et al., "Homology between cellulase genes of Trichoderma reesi: complete nucleotide sequence of the endoglucanase I gene", pages 253-263, see entire document.	1-74
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	FEMS, Symp. 43, issued 1988, J. Knowles et al. "The use of gene technology to investigate fungal cellulolytic enzymes Trichoderma reesi cellulase complex gene cloning and expression in Saccharomyces cerevisise", pages 153-169, see abstract only.	1-74
Y	Abstracts of the Annual Meeting of the American Society of Microbiology, 85 Meeting, 193, issued 1985, K. Murphy-Holland et al. "Secretion activity and stability of deglycosylated cellulase of Trichoderma reesi- gene cloning" see abstract only.	1-74
Y	US,A, 4,894,338, (Knowles et al.) 16 January 1990. See entire document.	1-74
A,P	Archives of Microbiology, Vol. 155, No. 6, issued 1991, R. Messner et al., "Cellobiohydrolase II is the main conidial-bound cellulase in Trichoderma reesi and other Trichoderma strains", pages 601-606, see abstract only.	1-74
A	Current Genetics, Vol. 18, Issued 1990, F. Gruber et al., "Cloning of the Trichoderma reesi pyrG gene and its use as a homologous marker for a high-frequency transformation system", pages 447-51.	1-74
A,P	Biotechnology and Applied Biochemistry, Vol. 14, No. 3, issued 1991, E. M. Kubicek-Pranz et al., "Characterization of commercial Trichoderma reesi cellulase preparations by denaturing electrophoresis SDS-PAGE and immunostaining using monoclonal antibodies", pages 317-323, see abstract only.	1-74